CRANFIELD UNIVERSITY

GONÇALO LEITE

Potential for control of spoilage and mycotoxigenic species using mixtures of anti-oxidants, aliphatic acids and molecular approaches using RNAi

CRANFIELD HEALTH

PhD THESIS

Academic Year: 2012 – 2013

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Abstract

In recent years, consumer perceptions are that they would like minimum levels of preservatives or even preservative free food. However, this leads to higher risks of microbial spoilage problems, especially due to growth of spoilage fungi, which are capable of growth at intermediate environmental conditions. Studies have been carried out to evaluate the effect of different preservatives at optimal and sub-optimal concentrations on growth, biosynthesis of mycotoxin production at a molecular and phenotypic level for *Fusarium graminearum* (*Tri5*) and trichothecene production and *Penicillium verrusocum* (*otapks*Pv) and ochratoxin A (OTA) production. These were complimented by studies on development of RNAi approaches to inhibit key regulatory genes in the biosynthetic pathways for mycotoxins in these two species. Additional studies were carried out to develop a rapid technique for RNA extraction from fungal biomass.

Initial liquid media based studies identified the growth boundaries of a range of 20 spoilage fungi including 3 mycotoxigenic species in relation to preservatives and pH. This showed that up to the legal allowable concentrations of sorbic and benzoic salts at pH 3.0 all strains were capable of growth after 24h. With the exception of *F. graminearum* all the other species and strains of spoilage fungi were able to grow in these conditions. The use of a mixture of preservatives, a common practise in the food industry, proved effective at inhibiting growth of most spoilage fungi for 21 days at the EU legal limits. Over the EU legal limit of 250 ppm of potassium sorbate mixed with 150 ppm of sodium benzoate only *Aspergillus niger* had observable growth. Mixtures of weak organic acids, fumaric and malic acid, with the preservative potassium sorbate was shown to be effective at inhibiting growth below the legal limits of use of these food additives, even though the presence of potassium sorbate appears to be fundamental to the inhibition effect, of these natural food additives. Moreover the presence of fumaric acid stimulated growth of *Aspergillus flavus.* The extraction of high quality total RNA from low amounts of mycelium showed that up to 3 times higher yields can be achieved while improving RNA integrity and overall quality. This development also reduced the time required to extract fungal RNA and the risk of

cross-contamination showing the potential use in high throughoutput gene expression studies.

In vitro and *In situ* studies demonstrated the risk of using single sub-optimal antifungal compounds to inhibit growth and mycotoxin production. For *F. graminearum*, while growth was reduced, the *Tri5* gene expression and trichothecenes type B production were stimulated in the presence of thyme essential oil, Prochloraz and BHA. This was also shown with *P. verrucosum* where *otapks*Pv gene expression and OTA production were stimulated at different water conditions by the presence of sub-optimum concentrations of thyme essential oil and Prochloraz. The antioxidant BHA was able to reduce both *otapks*Pv expression and OTA production in *P. verrucosum*.

The use of siRNA oligonucleotides to silence *Tri5* and *otapks*Pv demonstrated that both *F. graminearum* and *P. verrucosum* possess the RNAi pathway machinery. In both mycotoxigenic fungi the expression of the target key biosynthetic pathway was knocked down. The optimum levels of the designed siRNA molecules were of between 10 and 25 nM for the molecules targeting *P. verrucosum otapks*Pv. Even though gene silencing using siRNA molecules is transient the effect on *otapks*Pv was still observable after 15 days. This lead to a 3 to 5 times a reduction in the amount of OTA. On the other hand, the silencing of *Tri5* in *F. graminearum* was only detectable in the first 6 days after transfection.

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1 Introduction

1.1 General Introduction

Food security is a major challenge for the beginning of this millennium. Food security is defined by the Food and Agriculture Organization (FAO) of the United Nations (UN) as access by "…all people, at all times, (…) to sufficient, safe and nutritious food to meet their dietary needs and food preferences for an active and healthy life." In despite the observed annual increase in food production over the last decades the number of undernourished people has been steady in the 800 million (FAO, 1996). It is estimated to be around 12% of the world population during this decade (FAO, 2012).

Diverse factors contribute to this reality, in spite of all the social economic factors that contribute to food insecurity, food safety is a key factor. An annual loss of 25% of the world's food production is estimated to be loss due to spoilage. Additionally 5 to 10% of food losses can be realistically attributed to fungal spoilage (Pitt and Hocking, 2009). Food safety has a direct impact on human health with foodborne disease accompanied by waterborne disease being responsible for the death of around 2.2 million people annually (WHO, 2013). Overall, it is estimated that over the last 60 years approximately 30% of all emerging infectious disease were caused by pathogens commonly transmitted through food (Jones *et al.*, 2008). The World Health Organization (WHO) and FAO came together creating in 1963 the Codex Alimentarius commission that nowadays covers about 99% of the world's population with its international food standards, guidelines and codes of practise intended on improving food safety.

Fungal spoilage can be found throughout the food processing chain, from field contamination to the consumers' house. Fungal spoilage by yeasts or filamentous fungi causes major economic problems to the food industry (Brul and Klis, 1999). Fungal spoilage, besides causing visual impact on food, also produces changes in the physicochemical and organoleptic properties of the food product. This may in some circumstances, cause serious health hazardous, due to the production of mycotoxins (Pitt and Hocking, 1997).

Chapter 1

Mycotoxigenic filamentous fungi have the capacity to grow in a wide range of conditions and depending on the conditions imposed the mycotoxin biosynthesis pathway can be fully activated or completely inhibited. The most important factors controlling fungal growth in foodstuffs are temperature, water activity (a_W), pH or intergranular gas composition depending on the food matrix in question (Magan, 2007; Magan and Aldred, 2007; Magan *et al.*, 2010).

Mycotoxins are toxic secondary metabolites that appear not to be required by the mycotoxigenic fungi for its normal growth or metabolism. *Aspergillus, Fusarium* and *Penicillium* are the main genera of mycotoxigenic fungi. The most relevant mycotoxins to human health and food safety are aflatoxins, fumonisins, ochratoxin A and trichothecenes. Mycotoxin effects can vary from acute to chronic toxicity. They can act as hepatotoxins, nephrotoxins, neurotoxins or even as immunosuppressants. Extended exposure and chronic toxicity is of special concern since most of them are classified as teratogens, mutagens and carcinogens. Mycotoxins are difficult to eliminate making it critical to control contamination levels in raw materials.

It is estimated that worldwide 25 to 50% of harvested crops are contaminated with mycotoxins. This percentage rises up to a staggering 80% in tropical regions (Konietzny and Greiner, 2003). The annual economic losses have not been accurately estimated but from mycotoxin contamination of wheat, maize and peanuts alone represents almost $1 billion (Dohlman *et al.*, 2003).

Fungal contamination can occur at all stages of food production. It is imperative to be able to identify food spoilage as early as possible and the microbial agent causing spoilage. In the case of mycotoxigenic fungi the ability to do so can significantly reduce the risk of mycotoxin contamination in the food chain. Several polymerase chain reaction systems have been developed for identifying fungi in food matrices (Paterson, 2006). More recently it has become important not only to detect mycotoxigenic fungi but to be able to assess mycotoxigenic gene expression and infer effects on mycotoxin biosynthesis pathway activation. Real time quantitative reverse transcriptase PCR (RT-qPCR) has the ability to quantify mRNA transcripts from key pathway genes.

Good Agricultural Practices (GAP) and Good Manufacturing Practice (GMP) under Hazard Analysis Critical Control Point (HACCP) principles should be considered when aiming to reduce mycotoxin contamination in the food chain. Starting from the planting stage where crop rotation, soil preparation and seed choice are all important in developing resistance to fungi. Going through the pre-harvest phase it is important to avoid plant damage and have good agricultural practices to optimise crop quality. Harvesting is influenced by prevailing climatic conditions and sometimes it is important to manage the post-harvest phase effectively, including drying and good storage hygiene including appropriate preservation techniques. This minimises the risk of fungal contamination, spoilage and potential mycotoxin contamination (Codex Alimentarius).

Resistant seed cultivars to fungi have been developed using different approaches including breeding and genetic modification. One such approach could be the use of interfering RNA (RNAi). This approach has also attracted interest in relation to developing systems where the RNAi could be used to interfere with the biosynthetic pathways for mycotoxins of mycotoxigenic fungi during plant infection. This has been demonstrated *in vitro* for *A.flavus* and aflatoxin production (Abdel-Hadi *et al.*, 2011). Small interfering RNA (siRNA) molecules have the capacity to, in a sequence specific way, to degrade target mRNA transcripts and inhibit gene expression. If key mycotoxigenic biosynthetic genes can be inhibited it should be possible to minimise or eliminate specific mycotoxin biosynthesis.

1.2 Traditional Food preservation systems

Chemical preservatives and antifungals are becoming less attractive, due to the fact that some of the most commonly occurring microorganisms which give rise to spoilage have already shown some resistance to them (Pitt and Hocking, 1997), and legal regulations of the permitted concentrations have become stricter (Brul and Klis, 1999). Moreover, in recent years consumers have shifted their habits and are now demanding more “naturally” preserved (or preservative-free) products (Loureiro and Querol, 1999).

Although there exists a huge variety of traditional preservation systems, and they vary considerably in different cultures. However, despite different approaches, all of these preservation methods involve processes based on changing the food matrix characteristics to make it more difficult for colonisation by spoilage moulds. The prime targets to achieve this are the use of individual or combinations of environmental hurdles such as pH, water activity, nutrient availability, additives and modified atmosphere packaging. These approaches help to obtain the required shelf-life of a raw commodity or a processed food product. However, there is consumer pressure to reduce the use of the existing preservatives such as those based on aliphatic acids and their salts (sodium/calcium propionate, sorbates, benzoates). However, these are predominantly fungistats and the right concentrations must be used for efficacy. Sub-optimal concentrations may allow spoilage fungi and mycotoxin contamination risks to increase (Arroyo *et al.*, 2005; Schmidt-Heydt *et al.* 2007).

1.3 Current Preservation Systems

In 1995, Leistner and Gorris developed the concept of Hurdle technology. The general idea is that the combined effect of several inhibitory agents/processes are greater than one used individually, achieving a greater overall level of protection in a foodstuff. This technology attempts to provide two very important advantages: the microbial stability of the foodstuff in question, and to improve - or to better preserve - the organoleptic properties of the foodstuff, and, hence, consumer acceptability.

There are two opposing views in relation to the efficacy of combined preservatives. One suggests that there are interactive effects between preservatives/preservation systems (synergy), which results in a greater degree of preservation than that expected. The other suggests that the overall effect, although complex, is not more than additive. It has been suggested via the Gamma Hypothesis that combinations of preservatives or inhibitory components act independently of each other (Zwietering *et al.*, 1992). Assuming that the Gamma hypothesis is valid, then growth models for spoilage microorganisms can be constructed and used in the food industry, decreasing the expensive challenge tests and also reducing the time required for new product development.

Currently, the most often used preservation technique for delaying or preventing spoilage is the application of a combination of factors, which hinders microbial growth (Guynot *et al.*, 2004). The most widely used technique for preservation of foodstuffs involves the use of a combined treatment in which weak acids are applied and their efficacy enhanced by slightly decreasing the pH (Gould, 1996).

1.3.1 Weak acids as preservatives

Guynot *et al.* (2004) showed that benzoic, sorbic and propionic acids and their salts, individually, are the most commonly used weak acids in bakery systems preservation ensuring longer shelf-lives. In a food survey of soft drinks (Food Standards Agency, 2006), 150 drinks were collected from UK shops and besides benzene analysis, and the preservatives used were also detailed in each soft drink collected. This showed that in soft drinks the use of combined preservatives is predominant. Nonetheless, little information has been reported regarding the use of combined preservatives, as well as the combined effect of preservatives with other physical parameters, such as pH, on fungal spoilage species.

Weak organic acids such as acetic, benzoic, lactic, malic and sorbic, have also been incorporated into the food packaging itself, allowing prolonged effects due to gradual release into the food matrix as well as preventing surface growing; it also gives marketing advantages since it reduces the amount of preservative added to the product itself (Appendini and Hotchkiss 2002; Magan *et al.*, 2013).

The activity of these molecules is based upon their undissociated forms (Eklund, 1983). It has long been observed that the effect of weak acids, when used as antimicrobials, depend on the pH value, showing decreasing efficacy as this value increases (Simon and Beevers, 1952). The latter study, while reviewing other research work, showed that, in many cases, they had failed to calculate the amount of undissociated form of these weak acids molecules in inhibitory concentrations. As Eklund (1983) stated "hence it is not surprising that the view should be widely held that the undissociated molecule is the only toxic form of a weak acid or base" (Simon and Beevers, 1952). When the environmental pH is decreased below the pKa of the weak acid, the undissociated form of the weak acid predominates, this undissociated acid molecule is

more lipophilic than the corresponding dissociated molecule, and the acid molecule is then easily transported by diffusion into the cells cytoplasm. Thus, due to higher pH values within the cell, the molecule dissociates into its corresponding base and proton. The cell's internal pH value then decreases, directly inhibiting the glycolysis enzymes (Lück and Jager, 2000) and "forcing" the cell to actively excrete these protons, hence dispensing energy, causing growth to be inhibited (Lambert and Stratford, 1999).

(a) Sorbic acid

Sorbic acid (Figure 1.1) is an organic compound, which has the chemical formula $C_6H_8O_2$, used as a food preservative and first isolated from the unripe berries of the Rowan (*Sorbus aucuparia*). Sorbic acid and its salts - sodium sorbate, potassium sorbate and calcium sorbate - have the E numbers E200, E201, E202 and E203, respectively (Samson and Dijksterhuis, 2007). In aqueous solution this weak acid has a pK_a value of 4.76. Sorbic acid is a permitted preservative in the EU, and may be used in soft drinks at concentrations of up to 300 ppm (European Commission, 1995). On the other hand, in the US, the Generally Recognized As Safe (GRAS) status conferred to this preservative allows it to be used at higher concentrations (FDA, 2010). Sorbic acid (or its salts) is commonly used in alcoholic and non-alcoholic beverages, meat and fish, bakery products, spreads, sauces and salads (Stratford and Eklund, 2003).

In 1998, Stratford and Anslow suggested that sorbic acid has a membrane active compound role, making its activity less linked to the matrix pH, as other preservatives, such as benzoic and propionic acid, whose mechanism has already been described. The authors claim that sorbic acid acts as a membrane-disrupting compound. Previously, Reinhard and Radler (1981) had already shown that the respiration and fermentation of *Saccharomyces cerevisiae* was less affected by sorbic acid than growth, which lead them to assume that sorbic acid influenced the permeability and the cells membrane. Besides this activity, less linked to pH, sorbic acid also has other advantages. It has no taste - or less than that of other preservatives - and has a lower price (Guynot *et al.*, 2004). The minor taste of sorbic acid (and a slight burning taste at the back of the mouth) is only detectable at concentrations higher than 300 ppm.

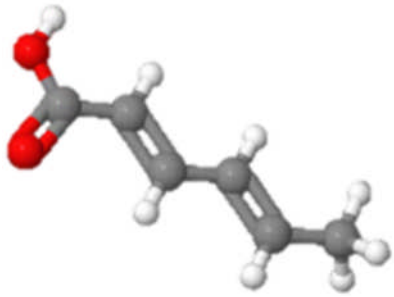

Figure 1.1 - Sorbic acid 3d structure (obtained from ChemSpider and adapted using jmol).

Various strains of moulds, such as some *Trichoderma* and *Penicillium* strains, as well as some yeast, are capable of detoxifying sorbates by decarboxylation, producing trans-1,3-pentadiene, which, characteristically, manifests as having a kerosene-like odour (Kinderlerer and Hatton, 1990). Plumridge *et al*. (2004) have shown that germinating conidiospores of *Aspergillus niger* can completely degrade any sorbic acid present to 1,3-pentadiene, enabling growth in the preservative depleted medium.

Regarding the biochemical aspects of this compound, the metabolism of 1-^{14}C-sorbic acid has been studied by Fingerhut *et al*. (1962). Sorbic acid was found to be excreted, almost completely, and most of the activity was found in the subcutaneous fat deposits and the organs lipids.

(b) Benzoic acid

Similar, to sorbic acid, benzoic acid (Figure 1.2) is a permitted preservative in Europe and the USA, and is generally applied within the same limits as sorbic acid. It is found in cranberries and cloves (Swartz and Medrek, 1968; Chicester and Tanner, 1972) and has a pK_a value of 4.19 (Smith, 1988).

The use of benzoic acid has been increasingly reduced, as it has been shown that under prolonged incubation with ascorbic acid, benzene is produced (Food Standards Agency, 2006). This formation is then increased by the presence of fruit and/or vegetable extracts. It has been suggested that the inhibitory action of sorbic acid over yeasts is higher than that of benzoic acid, but the latter is better in spoilage control at pH 3 (Sand and Kolfschoten, 1969). As a general rule, it can be considered that both sorbic and benzoic acid are roughly equal and additive in their antimicrobial effects.

It is a colourless crystalline solid, and is known as the simplest aromatic carboxylic acid ($C_7H_6O_2$) (Samson and Dijksterhuis, 2007). Sodium benzoate (E211), potassium benzoate (E212) and calcium benzoate (E213) are the mineral salts of benzoic acid (E210), and are all currently used as food preservatives. Typical values of benzoic acid, as a food preservative, range between 50 ppm and 100 ppm and the maximum levels are regulated by international law (European Comission, 1995; Codex Alimentarius, 2010).

Industrially, benzoic acid is prepared by partial oxidation of toluene with oxygen. This process is catalyzed by cobalt or manganese, and uses cheap raw materials, providing a high yield and is considered environmentally green (Oussi *et al.*, 1998).

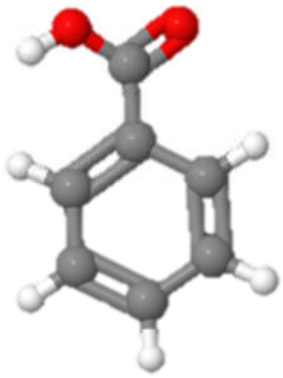

Figure 1.2 - Benzoic acid 3d structure (obtained from ChemSpider and adapted using jmol).

The transport of benzoic acid has been studied, using ^{14}C benzoic acid. The uptake occurs by rapid simple diffusion through the lipids of the plasma membrane (Gutknecht, 1984; Warth, 1989). In 1983, Kerbs *et al.* showed that benzoic acid accumulates as benzoate in the cytoplasm, lowering the cytoplasmatic pH.

Steels *et al.* (1999) showed that some yeasts are able to adapt to this preservative, by pre-growing them in low concentrations of benzoic acid. This must be taken into account while using this preservative, poor hygiene via splashing in the industrial environment can encourage the development of resistant yeast cells.

(c) Malic acid

Malic acid (E296; Figure 1.3) is a white or nearly white crystalline powder or granules, which as the chemical formula $C_4H_6O_5$, this organic acid can form stable complexes with metal ions being for that reason a chelating acidulant. It is a food additive that may be used in specific foods under the conditions of good manufacturing practices (GMP). It is also classified as GRAS status by the Food and Drug Administration (FDA, 2010) (Codex Alimentarius, 2010). Malic acid, via cytoplasmatic damage, was able to produce a 5 log reduction to foodborne bacterial pathogens; this is the minimal required reduction by the FDA for treatments, applied to commercial preparations of fresh juices, (Raybaudi-Massilia *et al.*, 2009). The cytoplasmatic damaged cells observed from the tested foodborne pathogens, is concurrent with the proposed general mechanism of action to weak acids.

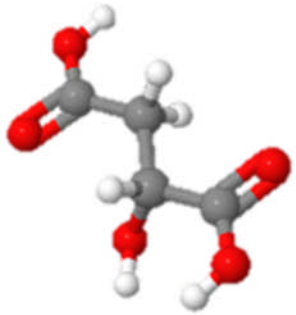

Figure 1.3 - Malic acid 3d structure (obtained from ChemSpider and adapted using jmol).

(d) Fumaric acid

Fumaric acid (E297; Figure 1.4) is an odourless white crystalline powder or granule, which has the chemical formula $C_4H_4O_4$, is a food additive that may be used in specific foods under GMP conditions (Codex Alimentarius, 2006).

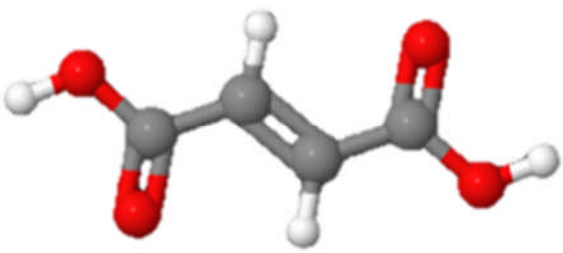

Figure 1.4 - Fumaric acid 3d structure (obtained from ChemSpider and adapted using jmol).

Most of the commonly used preservatives are fungistats and there is an increasing consumer pressure to reduce the use of such preservatives (Loureiro and Querol, 1999). Also, there is evidence that the use of sub-optimal concentrations of these preservatives (weak acids) stimulate growth and ochratoxin A production in *Penicillium verrucosum* (Arroyo *et al.*, 2005; Schmidt-Heydt and Geisen, 2007), and also growth in *Fusarium spp.* species (Marin *et al.*, 1999).

1.3.2 Natural preservatives

Food preservation science can potentially benefit from numerous antimicrobial agents, which are found as natural constituents of essential oils of herbs and spices that have been long known for their antiseptic properties.

Myers (1927) reported the use of essential oils, for their antiseptic properties, before the advent of antibiotics, being used to treat tropical yeast infections of workers in fruit canneries. Thyme, mustard, cinnamon and clove were found particularly effective. Maruzzella and Liguori (1958) surveyed a total of 110 essential oils and described 100 to have antimicrobial properties against both filamentous moulds and yeasts. In Table 1.1 are shown some other natural compounds already shown to have antimicrobial action that can, potentially, be used as food preservatives.

Table 1.1 - Natural compounds shown to have antimicrobial action.

Compound	Active component (if determined)	Reference
Thyme	thymol and carvacrol	Myers, (1927) Hulin *et al* (1998) Burdock, (2002)
Mustard	Oil of mustard	Ekanayake *et al.* (2002) Nielsen and Rios, (2000)
Cinnamon	Cinnamaldehyde	Myers, (1927) Conner, (1993) Faid *et al.* (1995) Burdock, (2002) Plumridge *et al.* (2008)
Cloves	Eugenol, eugenyl acetate and β-caryophyllene	Nielsen and Rios, (2000)
Garlic	Garlic oils	Nielsen and Rios, (2000)

These are some of the essential oils which have shown to have some potential as preservative aids or even as preservative agents in the food industry. Burdock (2002) names other essential oils, as potential preservatives, such as basil, lemongrass, peppermint, wintergreen and vanilla, even though Nielsen and Rios (2000) have not detected any type of inhibition by vanilla oil.

Almost without exception, essential oils constitute potent antimicrobials against fungi and bacteria. However, the components of these oils are chemically diverse. Some agents are aliphatic, some acids, some aldehydes. The common factor to all essential oil antimicrobial agents is that they are oil soluble, hydrophobic compounds. This makes it likely that membrane damage is, probably, the basis of the antimicrobial activity of these agents.

Both in Europe and the USA, essential oils are considered as natural flavours. The Food and Drug Administration (FDA) defines natural flavours as “the essential oil, oleoresin, essence or extractive, protein hydrolysate, distillate, or any product of roasting, heating or enzymolysis, which contains the flavouring constituents derived from a

spice, fruit or fruit juice, vegetable or vegetable juice, edible yeast, herb, bark, bud, root, leaf or similar plant material, meat, seafood, poultry, eggs, dairy products, or fermentation products thereof, whose significant function in food is flavouring rather than nutritional." (FDA, 2009).

As most flavours have GRAS status, they can be used at reasonable amounts and although legally they cannot be intended to preserve the product, that "side effect" is a plus for its application beside the only legal application that it enjoys, which is as a flavouring compound.

1.3.3 Antioxidants

Fungi, and, in particular, filamentous species, show high levels of resistance to chemical preservatives, as well as to low a_W and acidic environments (De Boer and Nielsen, 1995). Boutigny (2009) suggested that natural plant antioxidants would be able to inhibit mycotoxin biosynthesis, since lower concentrations of ferulic acid, a phenolic acid with strong antioxidant properties, have strongly reduced type B trichothecene production without reducing the fungal biomass. Studies by Aldred *et al.* (2008) compared essential oils and antioxidants for controlling growth and ochratoxin A by *P. verrucosum in vitro* and *in situ* in stored wheat. They found that resveratrol was much more effective than any of the essential oils examined. Thus knowledge of the relative mechanisms of action of essential oils and antioxidants could be of interest.

1.4 Fungi

Although, theoretically, all yeasts and filamentous spoilage fungi are capable of causing spoilage in low pH foods, relatively few are responsible for most cases of food spoilage. According to Pitt and Hocking (1997), while over one hundred species of yeasts were associated with foods, only about one tenth of these were responsible for food spoilage in foodstuffs. For filamentous fungi, a very limited mycobiota was found to be responsible for spoilage of different food categories (Filtenborg *et al.*, 1996).

Fungi are widely spread across a range of ecosystems. Their ability to cope with a wide range of environmental abiotic factors is one reason why this is possible. The capacity

to produce several metabolites and use extracellular enzymes allows them to use different nutrients that would not represent a primary source for other microorganism, gives them a potential edge over these others. .

The colonisation of ecological niches by filamentous fungi forces them to have several strategies to survive and prosper (Magan and Aldred, 2007). Fungi can grow with very limited resources even in bottles of aromatised mineral water (Nevarez *et al.*, 2009). A list of spoilage fungi typically causing problems in different food products are listed in Table 1.2.

Table 1.2 - Examples of food spoilage fungi and affected products (adapted from Brul and Klis, 1999).

Organisms	Products typically affected
Aspergillus versicolor	Bread, dairy products
Aspergillus flavus	Cereals, nuts
Aspergillus niger	Spices
Byssochlamys fulva	Cereals (airtight packs)
Fusarium oxysporum	Fruit
Neosartorya fischeri	Pasteurized foods
Penicillium roqueforti	Meat, eggs and cheese
Penicillium expansum	Fruits and vegetables
Penicillium commune	Margarines
Penicillium discolor	Cheese
Saccharomyces spp.	Soft drinks
Trichoderma harzianum	Margarines
Zygosaccharomyces bailii	Dressings

Although there are a large number of yeasts and filamentous moulds, only a small percentage of the total mycobiota is capable of giving rise to spoilage (Filtenborg *et al.*, 1996). Those non-spoilage species have been classified, by Davenport (1996) as Group III species. Spoilage species, in turn, are included in Groups I and II. The first group

includes the economically-dangerous spoilage species, which comprise all species of relevant yeasts. These are resistant to preservatives and a single cell can give rise to spoilage. Group II species are opportunistic ones, and include all filamentous fungi and some yeast species. Good Manufacturing Practices (GMPs) are required in order to prevent spoilage caused by these species, frequently present in factories. The non-spoilage species, Group III species, are good hygiene indicator species.

1.5 Toxic metabolites: mycotoxins

Mycotoxins are secondary metabolites produce by filamentous fungi. Secondary metabolites are small molecules not necessarily needed for the normal growth of filamentous fungi (Fox and Howlett 2008). From the hundreds of secondary metabolites known to be produced by filamentous fungi only a few pose significant problems as food contaminants, mainly aflatoxins, fumonisins, ochratoxin A and trichothecenes (Schmidt-Heydt and Geisen 2007).

The exposure to mycotoxins can result in either acute or chronic toxicity leading to health problems with several organs being affected or even death in the most severe cases (WHO, 2006). As a result the European Commission has been working on statutory limits and several regulations have arisen in recent years setting the maximum levels allowed in foodstuffs (European Commission, 2001; European Commission, 2005; European Commission, 2006; European Commission, 2007; European Commission, 2008). Mycotoxins are a global problem, thus around 100 countries, covering the majority of the world's population, has regulations or guidelines concerning some mycotoxins in foodstuffs (Egmond *et al.*, 2007).

Reverberi *et al.* (2010) while reviewing the biosynthesis of mycotoxins and their natural function suggested that mycotoxins are the fungal response to oxidative stress activated by different biotic or abiotic stresses or factors, or that at some level oxidative stress plays a pivotal role in mycotoxin synthesis, this new top to bottom approach to the whole mechanism might give new ways to explain why fungi produce such toxic compounds.

Mycotoxins are a food safety problem that needs to be addressed separately as mould growth and mycotoxin production are not always related; some cases of suboptimal concentrations of preservatives have been reported to stimulate mycotoxin production without the relative increase in mould growth (Arroyo *et al.*, 2005; Schmidt-Heydt *et al,.* 2007).

1.5.1 Aflatoxins

Aflatoxins are naturally occurring mycotoxins that are synthesized by species such as *Aspergillus flavus* and *Aspergillus parasiticus*. Indeed, aflatoxins represent a group of 18 types of compounds, the most common being B_1, B_2, G_1 and G_2, being aflatoxin B_1 considered the most toxic produced by both *A. flavus* and *A. parasiticus*, while G type aflatoxins (G_1 and G_2) are produced exclusively by *A. parasiticus*. These compounds have both carcinogenic and hepatotoxic activities, which depend, mainly, on the level and duration of exposure (Lewis *et al.*, 2005). In cases of ingestion of high levels of aflatoxins, acute aflatoxicosis may occur, manifesting as hepatotoxicity or, in more severe cases, fulminant liver failure (Fung and Clark, 2004).

1.5.2 Fumonisins

Fumonisins are mycotoxins synthesized by *Fusarium spp*., such as *Fusarium verticillioides* and *Fusarium proliferatum.* Until now, 28 types of compounds have been identified as fumonisins. They are divided as A, B, C and P. Fumonisins are an important cereal contaminant, especially in corn and corn- based products, especially FB_1, FB_2 and FB_3 are the most important (Yazar and Omurtag, 2008). Due to the structural resemblance to sphingoid bases, they are thought to disrupt the sphingolipid metabolism. Fumonisins have been epidemiologically linked to oesophageal cancer in humans (Wild and Gong, 2010).

1.5.3 Ochratoxins

Ochratoxin A (OTA) is an important mycotoxin that is synthesized by species of two genera, *Aspergillus* and *Penicillium*, such as *Penicillium verrucosum, Penicillium nordicum, Aspergillus melleus* and *Aspergillus ochraceus.* It was from a culture of this

last species, that in 1965 OTA was isolated for the first time, consequently the name (Van der Merwe *et al.*, 1965). Ochratoxin has three forms A, B and C (Figure 1.5). OTA is the most relevant and toxic of the group, is a rapidly absorbed toxin with slow excretion, and because this is the main route of elimination, OTA is an accumulative toxin (Ringot *et al.*, 2006). To date there are a number of toxicological effects that are attributed to OTA, mainly due to animal models, that mainly relate to nephrotoxicity and carcinogenicity, but also extend to neurotoxicity, immunotoxicity and teratogenicity. Although the entire mechanism seems not clear, at this moment; it appears that OTA interferes epigenetically with key biological factors via, protein synthesis inhibition, oxidative stress, modulation of transcription factor activities and interfering with cell signalling (Marin-Kuan *et al.*, 2008). Abiotic factors ranging from the presence of essential oils, organic acids, preservatives and extending to light cycles, appear to modulate the expression of OTA, without the corresponding effects on fungal growth or biomass (Arroyo *et al.*, 2005; Schmidt-Heydt *et al.*, 2007; Aldred *et al.*, 2008; Schmidt-Heydt *et al.*, 2010a).

The OTA biosynthetic pathway has not yet been fully revealed. Nevertheless, the OTA molecule is composed of a polyketide dihydroisocoumarin moiety linked via an amide bound to phenylalanine. A nonribosomal peptide synthetase is most likely to be involved in this step (Abbas, *et al.*, 2009). Recently, a gene enconding a nonribosomal peptide synthetase (NRPS) has been described as part of OTA biosynthetic pathway in *Aspergillus carbonarius*. The knockdown of this gene produced an interesting effect of increasing ochratoxin β, the dechloro analog of ochratoxin α which together with OTA were absent. This would suggest that the phenylalanine bound to the polyketide dihydroisocoumarin would precede the chlorination step of ochratoxin β and the NRPS would be somehow involved in this step (Gallo *et al.*, 2012). Labelling experiments showed that the phenylalanine moiety results from the shikimate pathway and the polyketide dihydroisocoumarin moiety arises from the pentaketide pathway (Ringot *et al.*, 2006). Furthermore the first step of the pentaketide pathway involves a polyketide synthase and is encoded in *P. verrucosum* by *otapks*Pv gene.

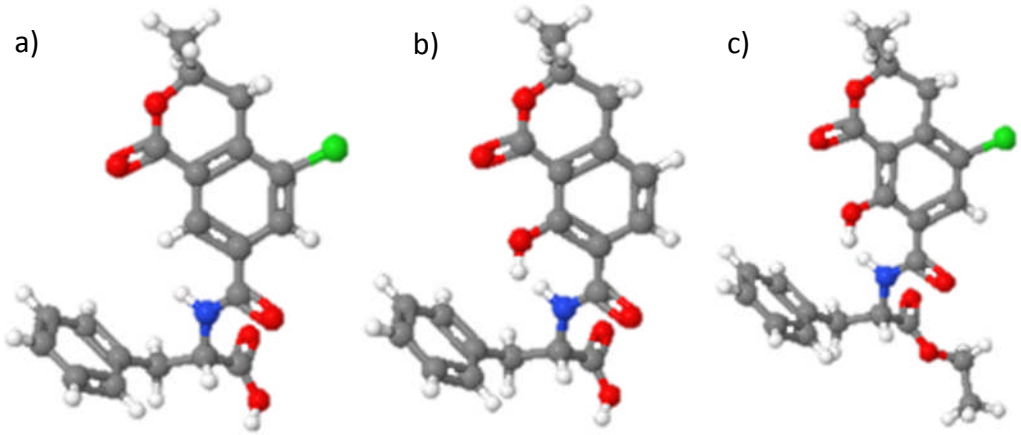

Figure 1.5 - a) Ochratoxin A 3D structure; b) Ochratoxin B 3D structure; c) Ochratoxin C 3D structure (obtained from ChemSpider and adapted using jmol).

Recently part of the gene cluster responsible for the biosynthesis of OTA in *Penicillium* has been reviewed by el Khoury and Atoui (2010). In *P. nordicum,* the cluster of 10 kb consist of three genes *otapksPN*, a polyketide synthase, which is different from PKS genes found in *Aspergillus* species and *otapks*Pv found in *P. verrucosum*. The second gene *npsPN*, has homology to non-ribosomal peptide synthetases, and the third *aspPN* has homology to fungal alkaline serine proteinases. While the biosynthetic pathway has not been fully understood a hypothetical OTA biosynthetic pathway as proposed by Huff and Hamilton (1979) is presented in Figure 1.6.

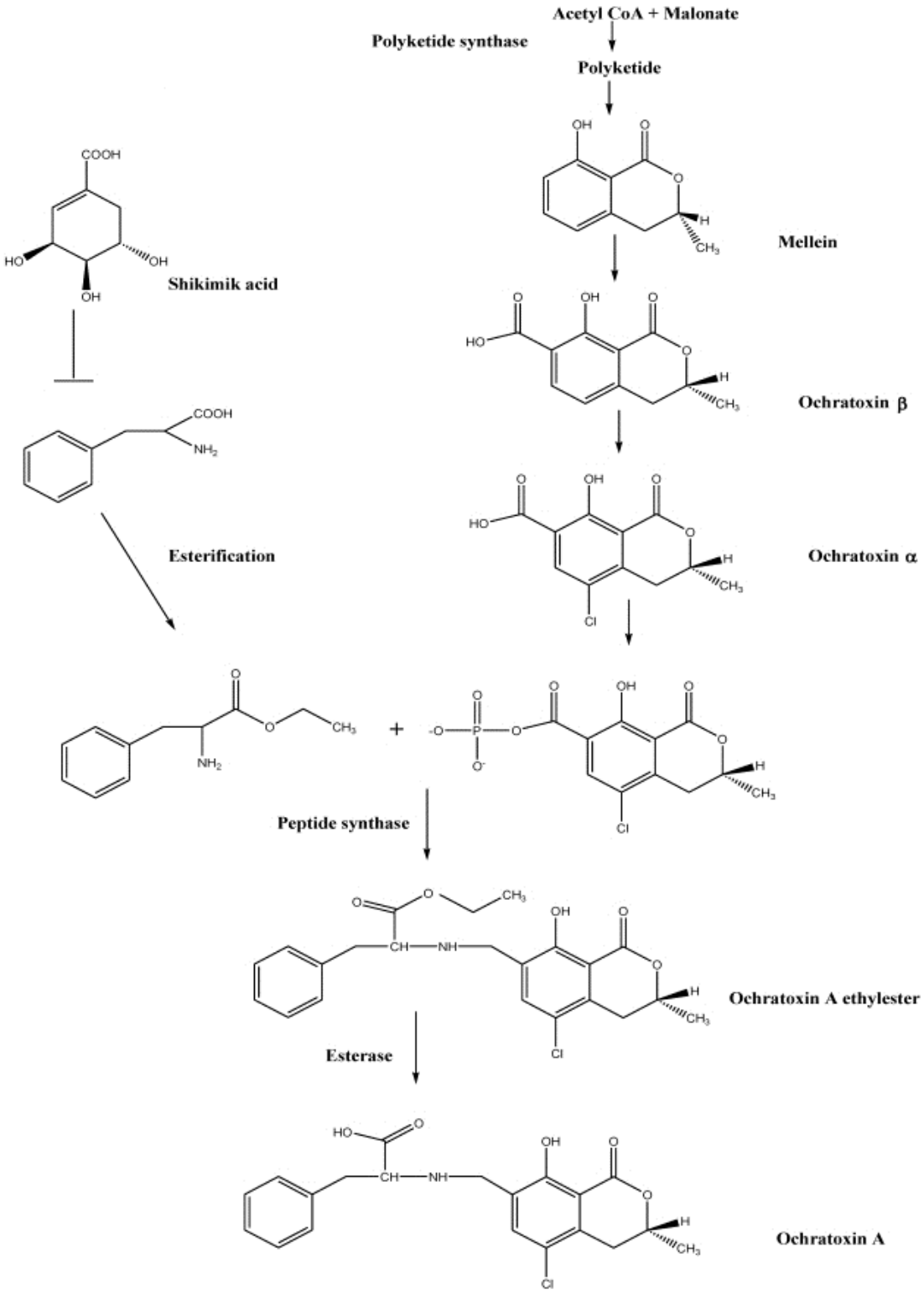

Figure 1.6 - Ochratoxin A biosynthetic pathway proposed by Huff and Hamilton (1979).

1.5.4 Trichothecenes

Trichothecenes are a large group of mycotoxins. They represent over two hundred secondary metabolites. The first member trichothecin was characterized in 1948 (Freeman and Morrison, 1948), irrespective of the description of several other members, this group of mycotoxins was only formed with the discovery of the common sesquiterpenoid skeleton structure in 1967 (Godtfredsen *et al.*, 1967; Figure 1.7). Trichothecenes are produced by a considerable number of mycotoxigenic fungi from the genera, *Cylindrocarpon*, *Cephalosporium*, *Myrothecium, Phomopsis, Stachybotrys, Trichoderma, Trichothecium* and *Verticimonosporium*, although the main genus that synthesizes trichothecenes is *Fusarium* (Bräse *et al.*, 2009). *Fusarium spp.* are particularly important producers of non-macrocyclic trichothecenes, from which important toxins like T-2, neosolaniol, nivalenol (NIV), diacetoxyscirpenol and deoxynivalenol (DON), are representatives (Bennett and Klich, 2003).

Trichothecenes affect protein synthesis, by binding to the ribosome site where peptidyl transferase, an aminoacyltransferase, would bind, effectively stopping the tRNA translation. Furthermore, Li and Pestka (2008) reported the cleavage induction of the 28S ribossomal RNA of peptidyl transferase center, and even though no enzymatic capacity was attributed, it was suggested that T-2 and DON, the trichothecenes used in this particular study, were upregulating the expression of cellular RNAses. Some of the most toxic known compounds to man originate within this group of mycotoxins (Grove, 2007).

This fact gave it a potential biological weapon application, and although never admitted by any army, in 1981, the U.S. Secretary of State Alexander Haig, offered substantiation for his allegation, that in Southeast Asia trichothecenes had been used as biological warfare agents, this became known as the yellow rain controversy. This was never proved and latter independent analyses were unable to detect trichothecenes from similar yellow rain samples (Desjardins, 2009), faeces of honey bees were the explanation for the visual effect (Nowicke and Meselson, 1984). Naturally occurring trichothecenes were the cause of the clinical symptoms, this

demonstrates that trichothecenes are an important human health problem in Southeast Asia (Evans, 1983).

Trichothecene biosynthetic pathway starts with the cycling of farnesyl pyrophosphate by action of trichodiene synthethase forming the characteristic sesquiterpenoid skeleton structure (Grove, 2007). Trichothecene toxins like T-2, nivalenol (NIV), and deoxynivalenol (DON) will require around 15 further biosynthetic steps. The majority of the enzymatic steps involved in the biosynthetic pathway have been described and the gene involved has been associated (McCormick et al., 2011; Figure 1.7).

The trichothecene biosynthetic genes in *F. graminearum* are clustered together. The 25 kb cluster is composed of 12 co-regulated genes with 10 of them being required for trichothecene biosynthesis located on chromosome 2. A further 3 additional genes, *Tri1*, *Tri101*, and *Tri15*, are located on chromosomes 1, 4, and 3, respectively (Hallen-Adams *et al.*, 2010).

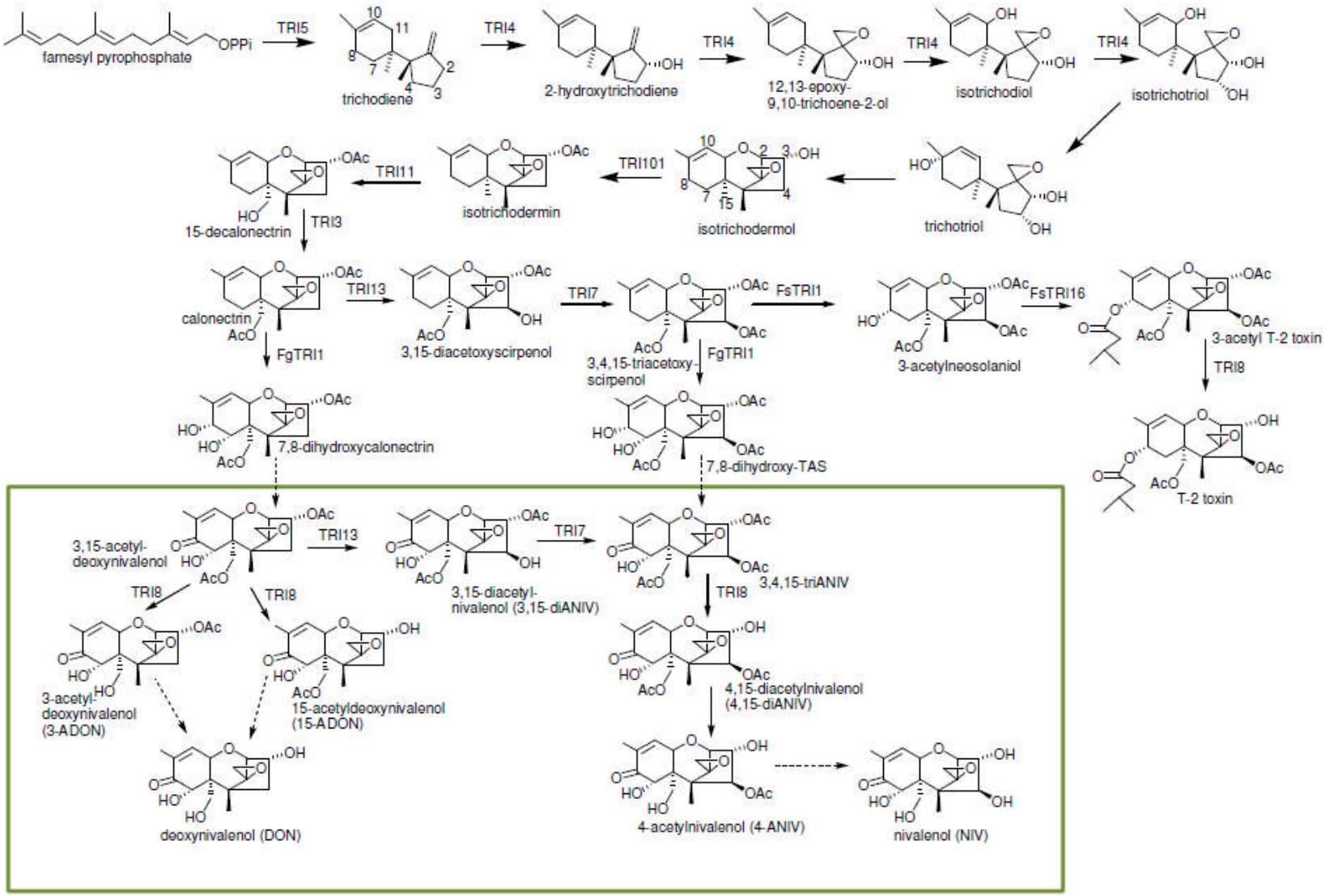

Figure 1.7- *Fusarium* trichothecene biosynthetic pathway proposed by McCormick *et al.* (2011). The genes encoding the specific enzymatic steps are next to the arrow, dashed lines represent steps without an identified gene. The green box represents type b trichothecenes.

1.6 RT-qPCR: quantifying gene expression

The enzymatic amplification of a specific target DNA region defined by two opposed oligonucleotides, called primers, complementary to opposite DNA strands, that, allied with the use of a thermostable polymerase enzyme, were the novelties presented by Mullis in 1986 and gave rise to PCR as we know it today (Bartlett and Stirling, 2003).

The chain reaction is achieved by a denaturation step, were the DNA strands and oligonucleotides are separated. Early polymerases were isolated from *E. coli* (Lehman *et al.*, 1958) and they would lose activity if subjected to the high denaturing temperatures required to open the double stranded DNA.

First synthesised oligonucleotides were developed by Khorana's group, early publications of Byrd *et al.* (1965) identified synthetic oligonucleotides as templates in DNA polymerase reactions. In fact, the second step of PCR uses synthetic oligonucleotides, complementary to opposite DNA strands. These oligonucleotides act as primers for the polymerase reaction, they signal the replication starting point and the direction, at the complementary position where they anneal to the DNA sequence. The melting temperature of the primer by definition is the temperature at which one half of the DNA duplex will dissociate to become single stranded. The annealing temperature will result from both primer and product melting temperatures using Rychlik *et al.* (1990) formula as follows:

$$T_a = 0.3 \times T_m(\text{primer}) + 0.7\, T_m(\text{product}) - 14.9$$

Equation 1.1 - Equation used for the calculation of the optimum annealing temperature of a set of primers (Rychlik *et al.,* 1990).

The polymerase reaction itself will follow the annealing step. During this step an optimum temperature is used for the DNA polymerase enzymatic reaction to occur which is around 72ºC for Taq polymerase that was isolated from *Thermophilus aquaticus* and its high temperature resistance, that tolerates the cycling between denaturation, annealing and extension without losing its efficiency, that allows the chain reaction to occur.

The reaction will produce a new copy of each strand per cycle, so it will double itself and it can be calculated using the formula 2^n were n is the number of cycles, assuming a reaction efficiency of 100%. This characteristic output can be used to quantify unknown starting number of copies. Real time qPCR will use a fluorescence probe or dye and a thermocycler capable of measuring florescence at each cycle.

1.7 RNAi: Discovering gene function

Schepers (2005) defined interference as "...the process in which waves (RNAs) of the same frequency (sequence) combine to reinforce or cancel (delete) each other", he also suggested that this approach may be effectively used to interfere with a specific target at a post-transcriptional level. This means that it may be possible to design gene probes which interfere with the biosynthetic pathway of the normal pathway. This can be used for mycology study. Fungal growth and metabolite production, including mycotoxins, could be targeted at the genomic level without the need to generate mutants with "knock-out" genes, enabling rapid genome wide studies in fungi (Nakayashiki, 2005).

When Fire *et al.* (1998) described that only a few molecules of double-strand RNA produced genetic interference in *Caenorhabditis elegans* they unveiled, the sought after potential to, in a sequence specific manner, knockdown a gene. *RNAi* was considered "The Breakthrough of the year 2002" by Science (Couzin, 2002). It is a powerful reverse genetic tool offering the possibility to study on a sequence-specific manner, gene function which for studying a gene family is useful, by using one conserved region of a gene family, it would be possible to silence several family members using a single RNAi (Nakayashiki and Nguyen, 2008).

On the other hand the promising high specificity of RNA interference was failing, in some studies microarray analyses clearly demonstrated off target silencing. This however is not yet fully understood, since genes with a better matching sequence sometimes are not the affected genes (Jackson *et al.*, 2003; Aagaard and Rossi, 2007). Several mechanisms have been suggested as responsible for these off target effects, some of them are related to high concentrations of siRNA and so they can be

eliminated by reducing the siRNA concentration. Others appear to be intimately related to the RNA interference pathway itself, and the way that the target gene is identified. A 7 nt complementary region on a siRNA target to GRK4 produced down regulation of HIF-1α. In this study, they found that genes with multiple partial complementary sites, at the 3'-untranslate region of the gene, to positions 2-8 nt at the 5' end of the antisense strand of the siRNA, would be potential off target genes (Lin *et al.*, 2005). In 2006 two different studies confirmed the importance of this "seed" region for the design of siRNA, to prevent off target silencing (Birmingham *et al.,* 2006; Jackson *et al.,* 2006). Both showed that the perfect match between the hexamer or the heptamer seed (the positions 2-7 or 2-8 of the antisense strand) of the siRNA and the 3'-UTR of one gene, would produce gene silencing. Moreover, even the delivery system used to carry the siRNA appears to change gene expression patterns (Fedorov *et al.*, 2005).

As the biosynthetic pathways and key regulatory genes for the production of several mycotoxins are known it would be very interesting to examine the potential for using the RNAi approach to reduce or inactivate key biosynthetic genes, thus inhibiting phenotypic production of the mycotoxin.

1.8 RNAi pathway

RNAi has been described in several eukaryotic organisms including animals, plants and fungi species. The mechanism appears nevertheless to be common to all these organisms. RNAi is activated by dsRNA or siRNA molecules and requires three preserved protein structures, RNA interference pathway is shown on Figure 1.8.

The silencing mechanism starts by the cleavage of dsRNA into smaller 21–23 nucleotides fragments analogous to the target gene. This initial step is carried out by an Rnase-III family member called Dicer. The cleavage of the dsRNA molecules is an ATP-dependent reaction and results into two types of smaller RNA molecules, microRNA (miRNA) and siRNA.

After the cleavage of the dsRNA, Dicer will be responsible for presenting the siRNA molecules to the other two required proteins. A dsRNA binding protein is required for

the recruitment of an Argonaut family protein. These will form a protein assembly defined as RNA-Inducing Silencing Complex (RISC). The RISC complex uses the antisense strand of the siRNA as a guide strand to degrade the corresponding mRNA, resulting in a sequence specific gene silencing (Yu *et al.*, 2002).

In addition endogenous kinase rapidly phosphorylates siRNA since a 5' phosphate is essential to enable RISC assembly. In humans the RISC assembly is composed of the binding protein TRBP required by the Dicer to recruit the Argonaut family protein, AGO2 (Chendrimada *et al.*, 2005). The complex unwinds the dsRNA and uses one of the ssRNA as a guide strand complementary to the mRNA of the gene of interest. Even though RISC assembly and siRNA is ATP independent the unfolding requires ATP. At this stage the target mRNA is cleaved at a single site 10 nucleotides from the phosphorylated 5' end of the siRNA (Dykxhoorn *et al.*, 2003). The gene silencing even though transient the effect appears to be amplified. The initial exogenous siRNA or dsRNA molecules can give origin to new molecules.

Furthermore short miRNA molecules also produced via Dicer cleavage are capable of causing translational silencing. This miRNA can either result from the generation of small hairpin RNA (shRNA) sequences in the nucleus from non-coding RNA or from maturing miRNA from pre-miRNAs at the cytoplasm serving these as the substrate for nuclear export (Lee *et al.*, 2002). miRNA unlike siRNA can have partial complementarity to the target mRNA and still cause gene silencing. This mechanism is achieved in a similar manner to the siRNA molecules.

RNA-induced transcriptional silencing (RITS) is also achieved using some of these complex proteins even though other PIWI Domain Proteins (PPD) are involved and the complex formed can contain a chromodomain protein leading to chromatin remodelling in the nucleus and transcriptional silencing (Dykxhoorn *et al.*, 2003).

RNA gene silencing, protects the genome against viruses and transposons, while removing abnormal nonfunctional mRNAs (Tijsterman *et al.*, 2002). The use of exogenous dsRNA is limited as longer than 30 nucleotides induce a sequence-nonspecific interferon response. This response causes the degradation of mRNA and

activates Protein Kinase-R (PKR), leading to mRNA translation inhibition (Dykxhoorn *et al.*, 2003).

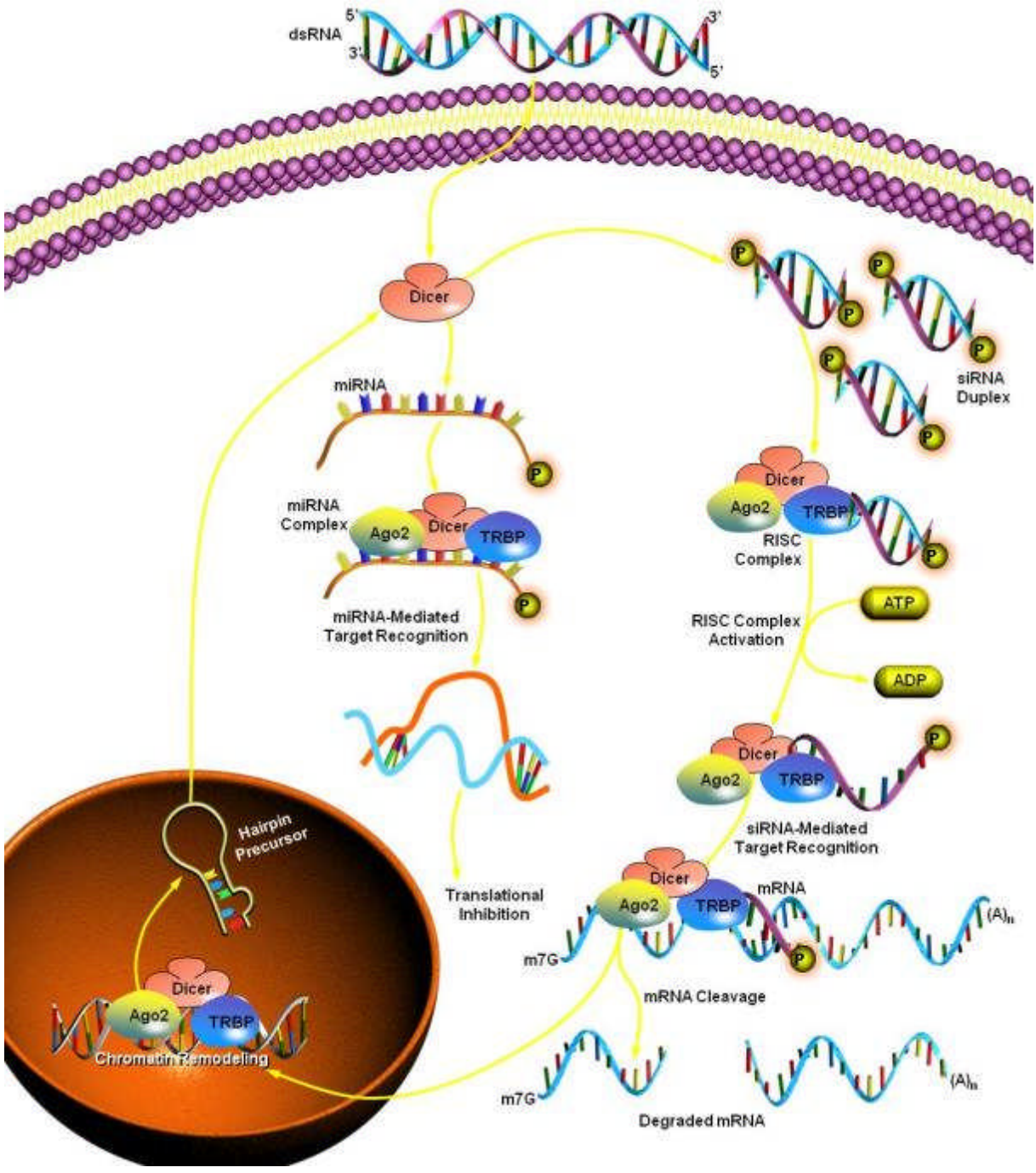

Figure 1.8 - Schematic representation of the RNA interference pathway, adapted from Pathway central, SABiosciences

1.9 Objectives and thesis structure

- Screening of spoilage yeasts and spoilage moulds for efficacy of a range of individual and mixtures of aliphatic acids for growth inhibition.
- Detailed studies on the potential for controlling growth of mycotoxigenic strains of *A. flavus*, *F. graminearum* and *P. verrucosum*.
- Development of a rapid technique for optimising RNA extraction from mycotoxigenic species fungal biomass.
- Effect of abiotic factors and comparison of antioxidant, fungicide and essential oil on growth and mycotoxin production by *F. graminearum* and *P. verrucosum*.
- Modulation of *Tri5 in F. graminaerum* and *otapks*Pv in *P. verrucosum* using RNAi.
- Potential for using RNAi approaches for controlling OTA and DON production by *P. verrucosum* and *F. graminearum*.

The thesis is thus divided in three different sections. In the first part the studies focussed on growth boundaries of spoilage fungi. The conditions when individual or mixtures of aliphatic acids as well as pH, temperature and natural preservatives were able to inhibit growth were determined (Chapter 2)

The second part of the thesis is formed by an initial method development (Chapter 3) and followed by dedicated chapters which deal with two different mycotoxigenic filamentous fungi. Studies on *Fusarium graminearum* (Chapter 4) and *Penicillium verrucosum* (Chapter 5) focussed on *in vitro* and *in situ* characterization of growth, mycotoxin production and monitoring of key biosynthetic pathway genes.

On the last part (Chapter 6) studies on mycotoxin control using RNAi were completed. The previously monitored genes were targeted using siRNA molecules and both gene expression and mycotoxin expression changes were quantified.

The overall aim was to identify possible and improved control strategies in different stages of the food chain and ultimately reduce mycotoxin contamination risk improving food safety.

The different components of the work that were carried out during this project are presented in the following schematic flow diagram (Figure 1.9).

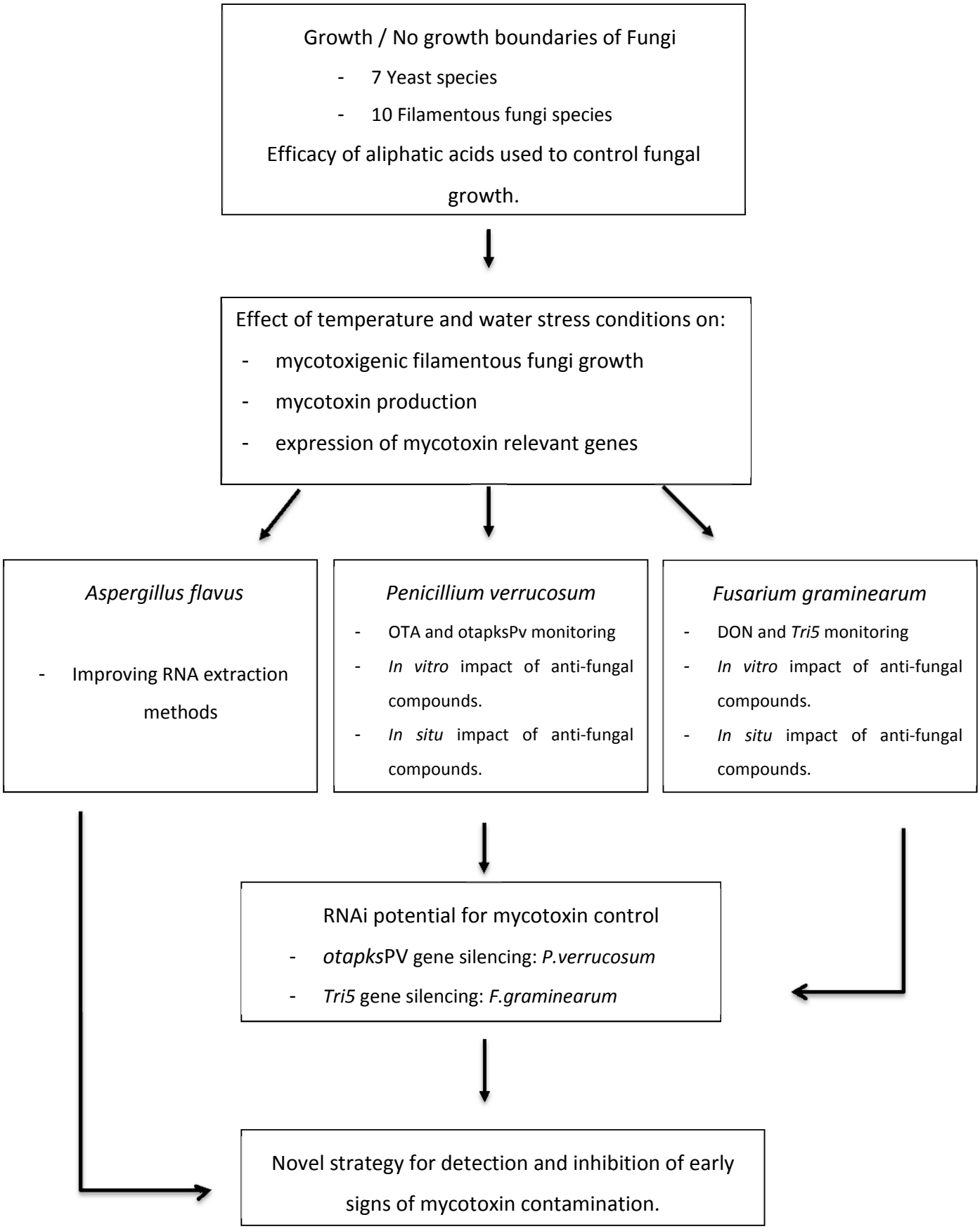

Figure 1.9 - Schematic flow diagram of the different components of this project.

2 Rapid screening of growth/no growth boundaries of potential spoilage fungi in relation to preservatives

2.1 Introduction

Fungal spoilage causes major losses and poses an important health problem due to mycotoxin contamination. The conditions in which fungal growth occurs are extensive. For this reason the adequate control strategies necessary to prevent fungal spoilage need to be effective in inhibiting growth.

The application of the hurdle concept currently is the most used preservation technique for delaying or preventing spoilage (Guynot *et al.*, 2004). The combined effect of several inhibitory agents/processes are greater than one used individually, achieving a greater overall level of protection in a foodstuff (Leistner and Gorris, 1995). The most widely used application of the concept for preservation of foodstuffs involves the use of a combined treatment in which weak acids are applied and their efficacy enhanced by slightly decreasing the pH (Gould, 1996).

The main targets to achieve this are the use of individual or combinations of environmental hurdles such as pH, water activity, nutrient availability, additives and modified atmosphere packaging. These approaches help to obtain the required shelf-life of a raw commodity or a processed food product. However, there is consumer pressure to reduce the use of the existing preservatives (Loureiro and Querol, 1999) such as those based on aliphatic acids and their salts (sodium/calcium propionate, sorbates, benzoates). However, these are predominantly fungistats and the right concentrations must be used for efficacy. There is little knowledge of how mycotoxigenic fungi cope with the preservation systems commonly use in foodstuff. Sub-optimal concentrations may allow spoilage fungi and mycotoxin contamination risks to increase (Arroyo *et al.*, 2005; Schmidt-Heydt *et al.*, 2007).

The use of chemical preservatives poses problems not only due to the consumers request for more naturally products, but also for its efficacy and safety. Some of the most commonly occurring microorganisms which give rise to spoilage have already shown some resistance to them (Pitt and Hocking, 1997). Food regulatory agencies regulate the compounds which can

be used in food products and the maximum levels that they are allowed to safeguard human health. Nevertheless as knowledge progress compounds believed to be safe are now under review. Additionally their use is being further limited by stricter legal regulations of the permitted concentrations (Brul and Klis, 1999).

The effect of combined preservatives can either by synergistic or antagonistic were the combined effect surpass, negatively or positively, the addition of each preservative individually or as suggested by the Gamma Hypothesis the overall effect, although complex, is not more than additive. In the last case the combinations of preservatives or inhibitory components act independently of each other (Zwietering *et al.*, 1992), as such growth models can be constructed and used in the food industry.

As a result, growth boundaries of important spoilage fungi can be important for the construction of growth models but also revealing the conditions at which growth is inhibited allowing stricter conditions for preserving foodstuff. Moreover the legal limits for preservative use is affected by the ability of some fungi, especially mycotoxigenic species to grow in the presence of such preservatives if they are not applied at the necessary required concentrations and affecting the relative risk of contamination (Magan and Aldred, 2007).

The objectives of this study was to (a) examine the effect of pH ranges for growth boundaries of a wide range of spoilage fungi, (b) evaluate the impact that single preservatives have on the growth boundaries and (c) measure the impact of preservative mixture in these growth boundaries.

2.2 Materials and Methods

2.2.1 Fungal species and isolates

The fungal species used included seven yeasts species and twelve species of filamentous fungi, including three mycotoxigenic strains. All the species used are listed in Table 2.1.

Table 2.1 - Summary list of fungal isolates used in these studies

Species		Remarks
Candida albicans	Yeasts	Isolate Y10
Candida parapsilosis		Isolate Y2
Candida tropicalis		Isolate Y9
Clavispora lusitaniae		Isolate Y13
Pichia membranifaciens		Isolate Y12
Saccharomyces cerevisiae		Isolate Y11
Zygosaccharomyces bailii		Isolate Y1
Aspergillus niger	Filamentous Fungi	Isolate Y14
Aspergillus puniceus		Isolate Y15
Fusarium oxysporum		Isolate Y3
Penicillium biourgeianum		Isolate Y6
Penicillium citreonigrum		Isolate Y5
Penicillium corylophilum		Isolate Y18
Penicillium echinulatum		Isolate Y8
Penicillium glabrum		Two different strains (Isolate Y4 and Y16)
Penicillium spinulosum		Isolate Y17
Aspergillus flavus	Mycotoxigenic Filamentous Fungi	Strain NRRL 3357
Fusarium graminearum		Strain I_3 L1-2/2D
Penicillium verrucosum		Strain OTA11

The species used are important food spoilage fungi. The isolates used coded with "Y" were isolated from food industrial environment and were kindly supplied by Dr Ronald Lambert, Cranfield University.

All the species where maintained on Malt Extract Agar (MEA, Oxoid, UK) at 25ºC. The cultures were sub-cultured onto Malt Extract Agar, at least three times before being used for an experiment.

2.2.2 Cells/Spores suspensions inoculum

From a 7 day fully-grown culture on MEA, spores of filamentous fungi and cells from yeasts cultures were collected using a sterile loop into sterile 25 ml Universal bottles containing 10 mL RO water with 0.05% Tween 80 and shaken well. The culture suspensions were counted using a haemocytometer. Using previously sterilized vials containing 10 mL of PBS prepared from tablets (Sigma-Aldrich, UK), suspensions of $1x10^6$ cells/spores per mL were prepared for each strain.

2.2.3 Media pH adjustment for determining how pH affects the growth of spoilage fungi

The effect of pH on the growth boundaries of the 18 species of spoilage fungi was assessed. In order to lower the pH to values more related to those found in beverages, HCl or KH-Phthalate buffer were used. The effect of pH and the adjusting agent on growth was also evaluated.

The Clark and Lubs solution (Bower and Bates, 1955) of KH-Phthalate buffer was prepared by adding 38.8, 22.3, 8.2 and 0.1 mL of a 0.1 M solution of HCl to 50 ml of 0.1 M solution of KH phthalate, adjusted using RO water to a total final volume of 100 mL, for a final pH of 2.5, 3.0, 3.5 and 4.0 respectively. To each, 100mL buffered solution, 0.5 ± 0.05g of Malt Extract (Oxoid, UK) was added. The other method used to adjust the final pH of the liquid media containing 0.5% of Malt Extract (Oxoid, UK), was by adding HCl, from a stock solution of 1 M, to the liquid media until the desired pH was obtained.

The difference between the measured and the desired pH was found to be < ± 0.05 in both methods. The liquid media was filter sterilized (0.2 µm filters, Sartorius, UK), 2.5 mL of each sterile pH adjusted media was added to each well of a sterile 24 well plate (Nunc, Denmark).

The plates were inoculated with 25 µl of the $1x10^6$ cells/spores per mL in 20 wells, individually; the remaining 4 wells were considered as blanks containing 2 mL of sterile media (Table 2.2).

Table 2.2 - Layout of the pH treatments used in the experimental system for testing how pH affects growth of individual test species. Y1, Y2, Y3, Y4 and represent individual species, M.C. represents media sterility control.

pH						
2.5	Y1	Y2	Y3	Y4	Y5	M.C.
3.0	Y1	Y2	Y3	Y4	Y5	M.C.
3.5	Y1	Y2	Y3	Y4	Y5	M.C.
4.0	Y1	Y2	Y3	Y4	Y5	M.C.

All plates were sealed with parafilm and incubated at 25ºC for 15 days.

To assess growth the plates were observed with the naked eye and with the help of a microscope, to determine whether mycelia growth had been initiated. This was determined by the presence of germ tube extension, fungal hyphae or sporulating structures in filamentous fungi and by cloudy media in yeasts.

Growth was evaluated after 1, 2, 3, 4, 5, 7, 10 and 15 days. After 15 days incubation, the pH of the medium from two yeast cultures and three filamentous fungi cultures, and the sterile control medium pH, was measured to determine pH shifts.

The experiment was replicated three times.

2.2.4 Preservative/weak organic acid solutions

Stock solutions of 100 000 ppm of sodium benzoate (BDH, UK) and potassium sorbate (Sigma ,Germany) were prepared, by diluting 10.0 ± 0.05 g of each in 100 mL of RO water. Working solutions were prepared by diluting 1 mL of these solutions in 100 mL, using RO water, this was then diluted to the desired test concentrations.

Stock solutions of 10 000 ppm of fumaric acid (Sigma, Germany) and malic acid (Fluka, USA) were prepared, by diluting 1.0 ± 0.005 g of each in 100 mL of RO water. Working solutions

were prepared by diluting 10 mL of these solutions in 100 mL, using RO water, this was then diluted to the desired test concentrations.

All stock solutions were filter sterilised (0.2 µm filters, Sartorius, UK) and kept at ambient temperature in the dark, until required.

2.2.5 Effect of single preservative/weak organic acid solutions on growth of test species

Sodium benzoate was used at 100, 200, 300 and 400 ppm; potassium sorbate at 50, 100, 150, 200 ppm. These concentrations are in accordance with the legal limit applied to these specific preservatives, when used by the beverage industry in Europe [300 ppm for sorbate and 150 ppm for benzoate (European Commission, 1995)].

The media with the respective concentrations of each of the individual preservatives was prepared by diluting 0.5% of Malt extract (Oxoid, UK) with the respective amount of preservative and then the pH was adjusted using HCl. The liquid media was filter-sterilized (0.2 µm filters, Sartorius, UK), 2.5 mL of each sterile pH adjusted media with preservative, was added to each well of the sterile 24 well plates detailed previously.

The plates were inoculated with 25 µl of the $1x10^6$ cells/spores per mL in 20 wells, individually; the remaining 4 wells remained with 2 mL of sterile media (Table 2.3 and 2.4).

Table 2.3 - Layout of the individual sodium benzoate treatments used in the experimental system for testing how individual preservative affects growth of individual test species. Y1, Y2, Y3, Y4 and represent individual species, M.C. represents media sterility control.

|Benzoate| (ppm)						
50	Y1	Y2	Y3	Y4	Y5	M.C.
100	Y1	Y2	Y3	Y4	Y5	M.C.
150	Y1	Y2	Y3	Y4	Y5	M.C.
200	Y1	Y2	Y3	Y4	Y5	M.C.

Table 2.4 - Layout of the individual potassium sorbate treatments used in the experimental system for testing how individual preservative affects growth of individual test species. Y1, Y2, Y3, Y4 and represent individual species, M.C. represents media sterility control.

|Sorbate| (ppm)						
100	Y1	Y2	Y3	Y4	Y5	N.C.
200	Y1	Y2	Y3	Y4	Y5	N.C.
300	Y1	Y2	Y3	Y4	Y5	N.C.
400	Y1	Y2	Y3	Y4	Y5	N.C.

All plates were sealed with parafilm and incubated at 25ºC for 15 days.

The growth was evaluated after 1, 2, 3, 4, 5, 7, 10 and 15 days. After 15 days of incubation, the pH, where growth was observed and in the sterile control, the medium pH was determined. The experiment was replicated three times.

2.2.6 Effect of mixtures preservative/weak organic acid solutions on growth of test species

Sodium benzoate was used at 100, 200, 300 and 400 ppm; potassium sorbate was used at 50, 100, 150, 200 ppm as detailed previously. There are no legal limits for fumaric and malic acid as food additives although they are limited to use in specific authorized foodstuffs (FDA, 2010).They were used at 125, 250, 500 and 1000 ppm.

Each mixture was prepared by adding the respective volume of preservative/weak organic acid together and then adjusting the volume to 100 mL using RO Water. The mixtures used are represented on Table 2.5.

Table 2.5 - Matrix of the relative concentrations of each preservative/weak organic acid used on the study of the growth boundaries of all spoilage fungi

|Benzoate| (ppm)	|Sorbate| (ppm)
50	100
50	200
50	300
50	400
100	100
100	200
100	300
100	400
150	100
150	200
150	300
150	400
200	100
200	200
200	300
200	400

|Fumaric| (ppm)	|Sorbate| (ppm)
125	100
125	200
125	300
125	400
250	100
250	200
250	300
250	400
500	100
500	200
500	300
500	400
1000	100
1000	200
1000	300
1000	400

|Malic| (ppm)	|Sorbate| (ppm)
125	100
125	200
125	300
125	400
250	100
250	200
250	300
250	400
500	100
500	200
500	300
500	400
1000	100
1000	200
1000	300
1000	400

To each individual mixture, 0.5 ± 0.05 g of Malt extract (Oxoid, UK) was added then the pH adjusted using HCl. The liquid media were filter sterilized (0.2 µm filters, Sartorius, UK). 2.0 mL of each sterile pH adjusted mixture media was added to a corresponding well within the sterile 24 well microplate.

Table 2.6 - Layout of the mixture sodium benzoate with potassium Sorbate treatments used in the experimental system for testing how mixture of preservatives affects growth of individual test species. Y1 represents one individual strain.

	|Benzoate| (ppm)					
|Sorbate| (ppm)	50	100	150	200	50	100
100	Y1	Y1	Y1	Y1	Y1	Y1
200	Y1	Y1	Y1	Y1	Y1	Y1
300	Y1	Y1	Y1	Y1	Y1	Y1
400	Y1	Y1	Y1	Y1	Y1	Y1

Table 2.7 - Layout of the mixture potassium sorbate with fumaric acid treatments used in the experimental system for testing how mixture of weak organic acids affects growth of individual test species. Y1 represents one individual strain.

	Fumaric acid (ppm)					
|Sorbate| (ppm)	125	250	500	1000	125	250
100	Y1	Y1	Y1	Y1	Y1	Y1
200	Y1	Y1	Y1	Y1	Y1	Y1
300	Y1	Y1	Y1	Y1	Y1	Y1
400	Y1	Y1	Y1	Y1	Y1	Y1

Table 2.8 - Layout of the mixture potassium sorbate with malic acid treatments used in the experimental system for testing how mixture of weak organic acids affects growth of individual test species. Y1 represents one individual strain.

	|Malic acid| (ppm)					
|Sorbate| (ppm)	125	250	500	1000	125	250
100	Y1	Y1	Y1	Y1	Y1	Y1
200	Y1	Y1	Y1	Y1	Y1	Y1
300	Y1	Y1	Y1	Y1	Y1	Y1
400	Y1	Y1	Y1	Y1	Y1	Y1

The plates were inoculated with 25 µl of the $1x10^6$ cells/spores per mL in 20 wells, individually; the remaining 4 wells remained with 2 mL of sterile media (Tables 2.6, 2.7 and 2.8).

All plates were sealed with parafilm and incubated at 25ºC for up to 21 days.

The growth was evaluated after 1, 2, 3, 4, 5, 7, 10, 15 and 21 days, when the species where subjected to the mixture of preservatives benzoate and sorbate. When the species where subjected to the mixture of the preservative sorbate and fumaric and malic acid, the growth was assessed for only 15 days. The experiment was replicated three times.

2.3 Results

2.3.1 Effect of pH and pH buffering system on the growth boundary of spoilage fungi

The percentage of cultures that displayed growth was calculated for all 20 isolates and all replicates, a total of 60 samples were examined. Table 2.1 shows the percentage of growth, when the pH was adjusted using a KH-Phthalate buffer.

Table 2.1 - Percentage of observed growth on all cultures using KH-Phthalate buffer to adjust the pH to the desired values.

pH	Time (Days)							
	1	2	3	4	5	7	10	15
2.5	43%	60%	60%	60%	60%	60%	60%	60%
3.0	65%	77%	82%	82%	83%	83%	83%	87%
3.5	95%	100%	100%	100%	100%	100%	100%	100%
4.0	100%	100%	100%	100%	100%	100%	100%	100%

The medium buffered with KH-Phthalate at pH 2.5, inhibited growth of 44% of the cultures, which represent all of the replicates for 20 different species, for the duration of the entire study, even at pH 3.0, there was still inhibitory activity after 15 days (see Appendix A1 for details). Table 2.2 shows the percentage of growth, when the pH was adjusted using a 1 M HCl.

Table 2.2 - Percentage of observed growth on all cultures using HCl to adjust the pH to the desired values.

pH	Time (Days)							
	1	2	3	4	5	7	10	15
2.5	75%	85%	90%	95%	95%	95%	95%	97%
3.0	98%	100%	100%	100%	100%	100%	100%	100%
3.5	100%	100%	100%	100%	100%	100%	100%	100%
4.0	100%	100%	100%	100%	100%	100%	100%	100%

The liquid media with adjusted pH showed that at pH 2.5 only *Saccharomyces cerevisiae* did not display observable growth after 15 days, at pH 3.0 only on one of the replicates of the same culture was not possible to detect growth after 24h incubation after 48h it was

possible to observe growth on all species and replicates. There was no inhibition effect at pH 3.5 and pH 4.0.

Table 2.3 shows the HCl adjusted medium pH, after 15 days with growth the sterility control media. The control media pH remained stable even after 15 days, nevertheless there was a slight decreased of the pH, on the higher pH media, after growth.

Table 2.3 - Measured pH before and after incubation for 15 days at 25ºC.

Initial pH	Control medium pH	Final pH
2.52	2.49	2.55
3.05	3.03	3.10
3.52	3.50	3.35
3.98	3.93	3.71

The pH was measured by filter sterilising (0.2 µm filters, Sartorius, UK) media from 5 replicates within cultures of filamentous fungi and yeasts that were fully-grown.

2.3.2 Effect of single preservative/weak organic acid solutions on growth of spoilage fungi

The use of single preservatives was capable of delaying the growth of several species tested; yeast species appear to be particularly sensitive, detailed data in Appendix A2. Even though, after 7 days growth was visible on all replicates at all concentrations tested (Figure 2.1 and 2.4).

Sodium benzoate, appear to be more effective at the same concentrations then potassium sorbate, nevertheless at the legal limit the inhibition effect was similar (Figure 2.1 and 2.2).

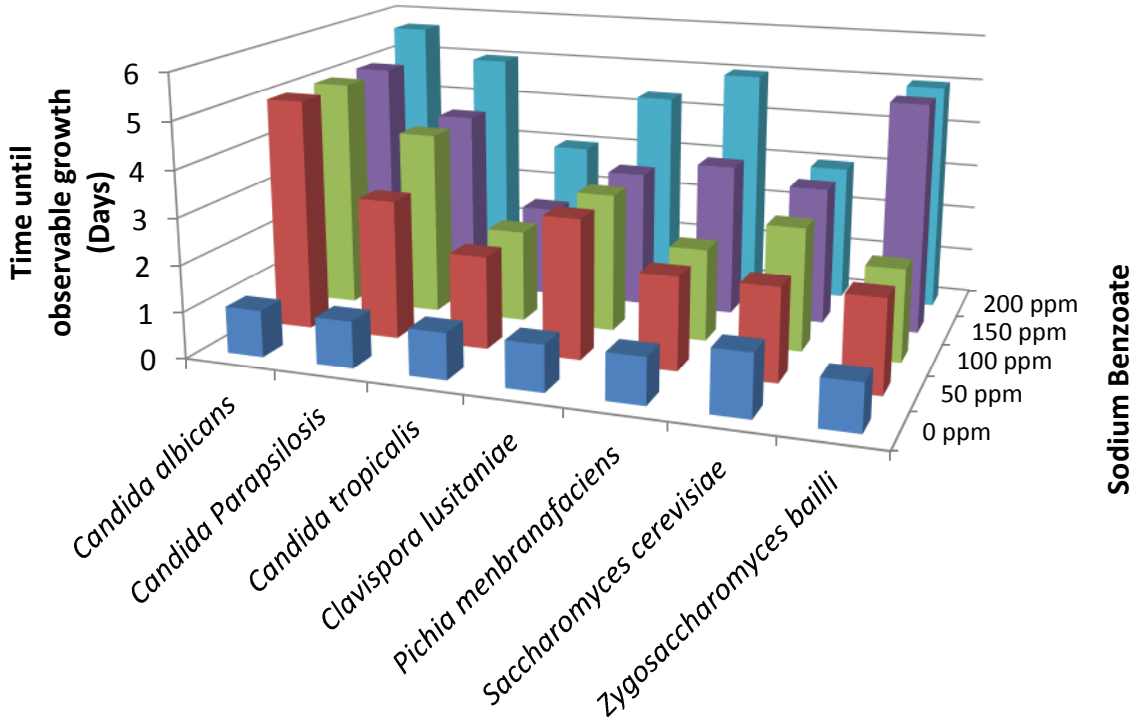

Figure 2.1- Effect of sodium benzoate on the time to visible growth of all yeast species.

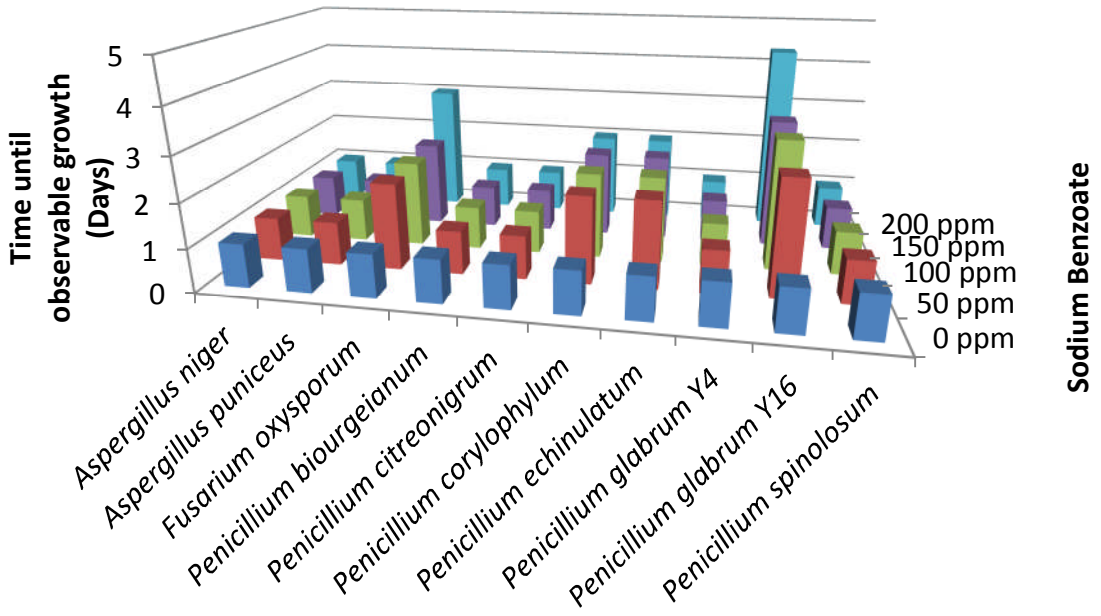

Figure 2.2 - Effect of sodium benzoate on the time to visible growth of filamentous fungi.

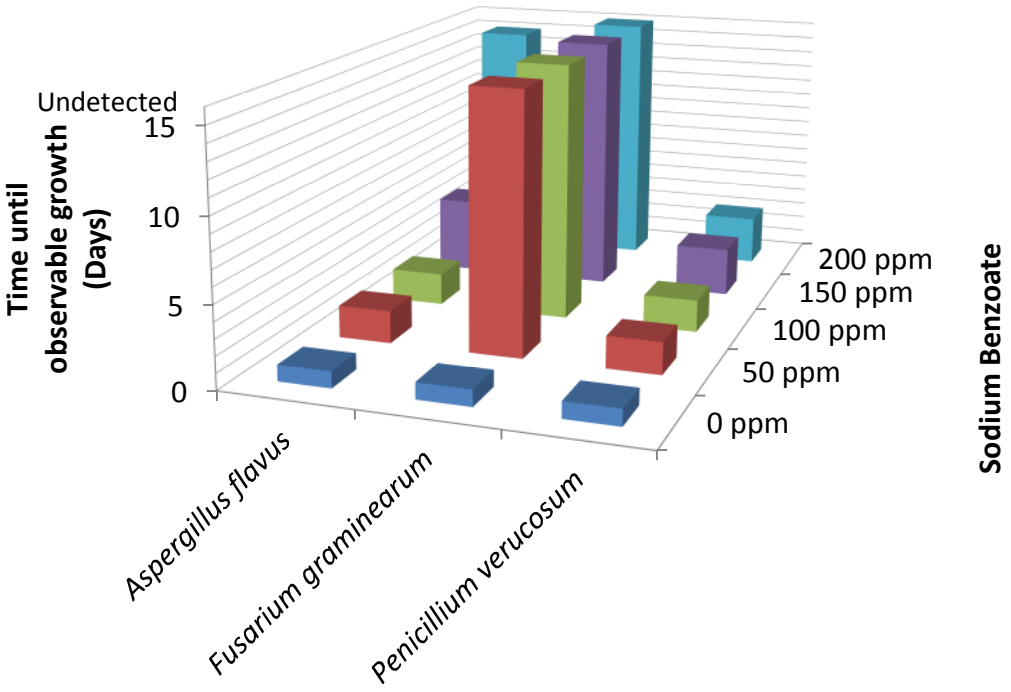

Figure 2.3 - Effect of sodium benzoate on the time to visible growth of mycotoxigenic filamentous fungi species.

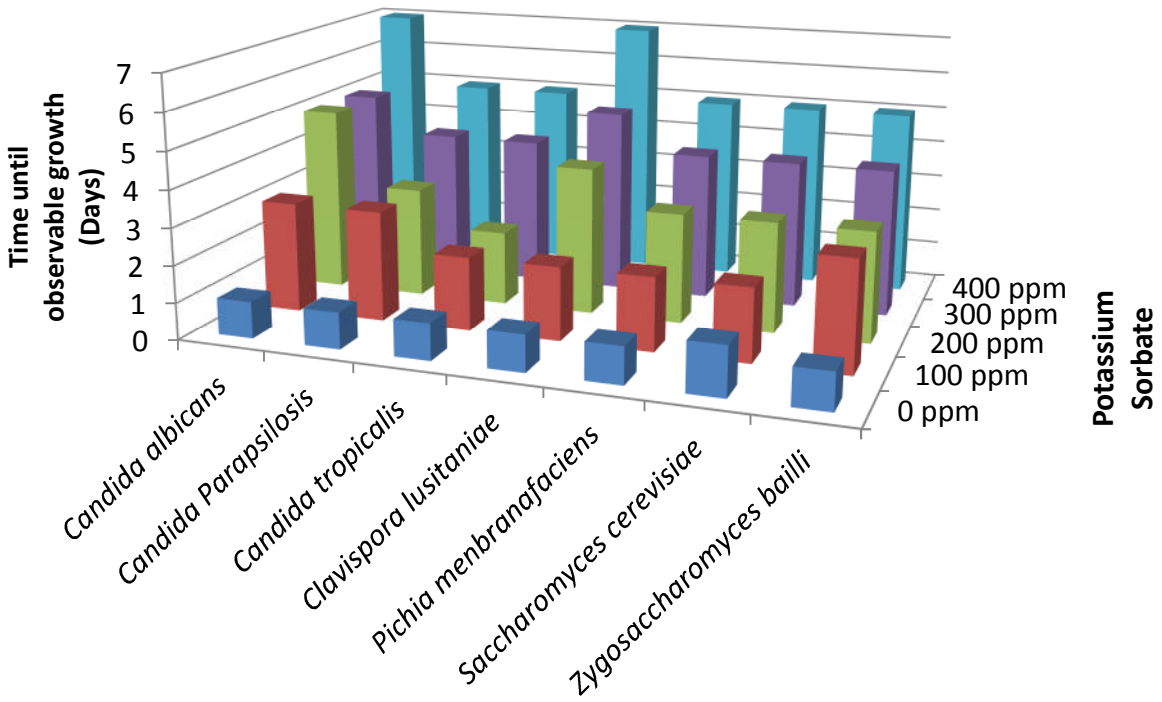

Figure 2.4 - Effect of potassium sorbate on the time to visible growth of all yeast species.

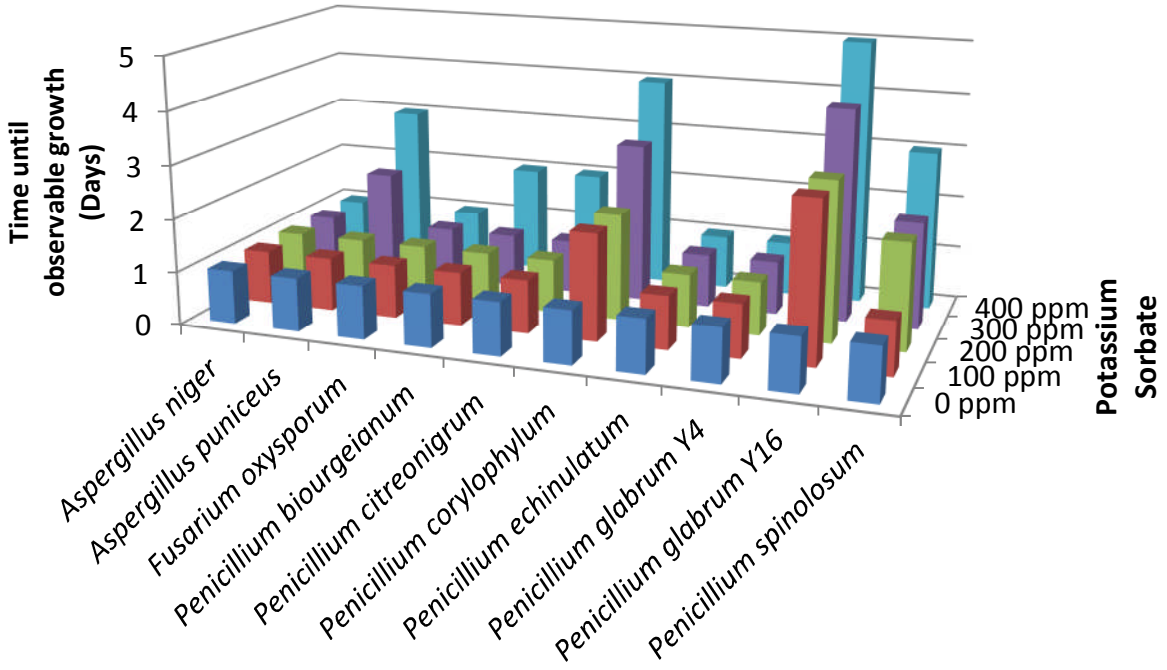

Figure 2.5 - Effect of potassium sorbate on the time to visible growth of filamentous fungi.

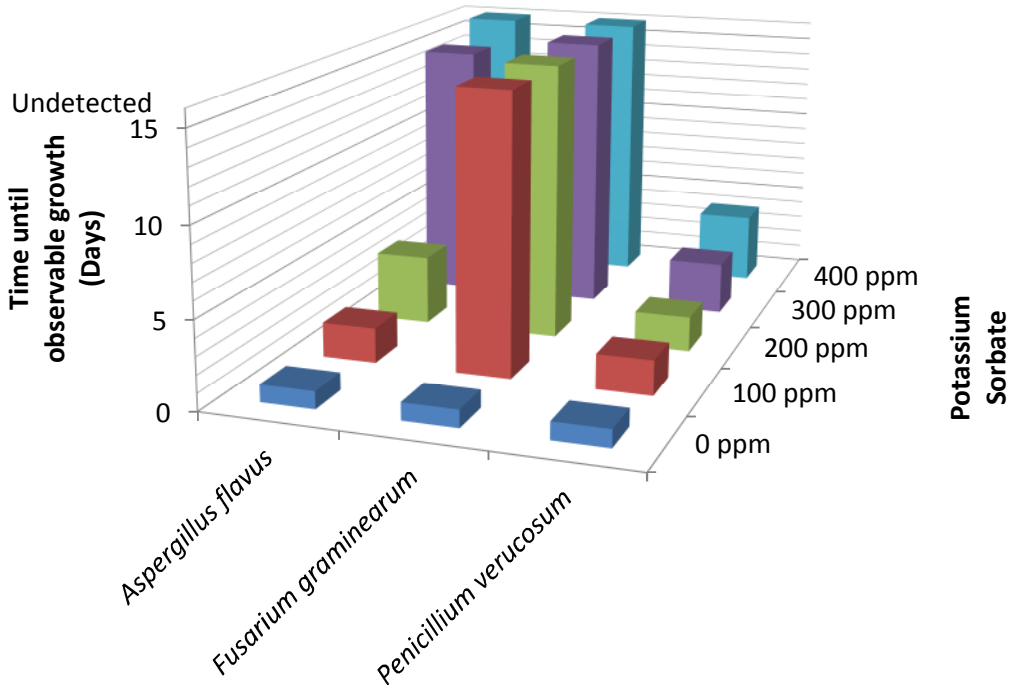

Figure 2.6 - Effect of potassium sorbate on the time to visible growth of mycotoxigenic filamentous fungi species.

2.3.3 Effect of mixture of preservative/weak organic acid solutions on growth of spoilage fungi

The mixture of preservatives effectively inhibited growth of all species tested, however at the EU legal limit, 250 ppm of potassium sorbate and 150 ppm of sodium benzoate, *Aspergillus niger* was still able to grow. All replicates of *A. niger* were able to grow after 21 days on a medium with 300 ppm of potassium sorbate and 150 ppm of sodium benzoate, detailed data in Appendix A3 (Figure 2.7).

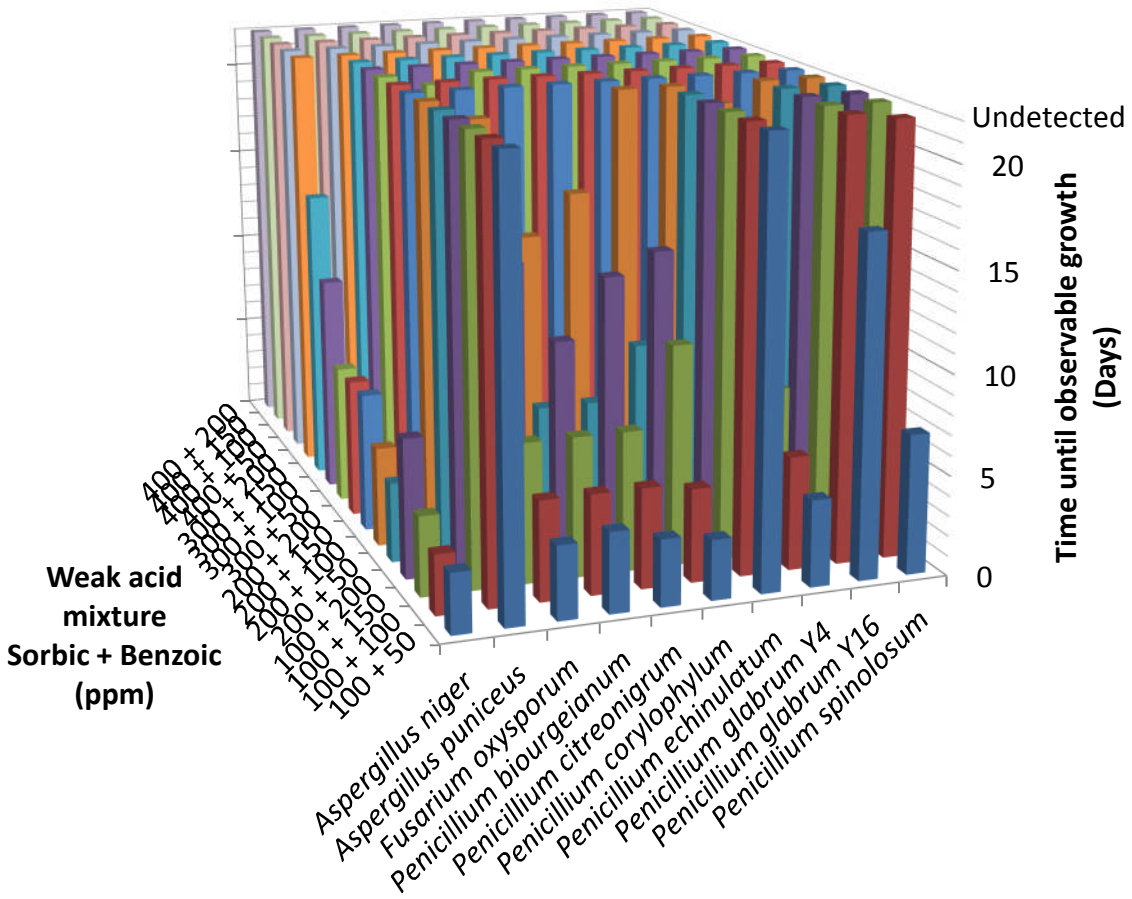

Figure 2.7 - Effect of mixture of potassium sorbate and sodium benzoate on the time to visible growth of filamentous fungi.

All other tested species were inhibited below the legal limit. *A. flavus* was the only mycotoxigenic specie capable of growth in the presence of potassium sorbate and sodium benzoate mixture. In fact, it was the only specie beside *A. niger*, capable of growth on media

containing >200 ppm of potassium sorbate (Figure 2.8). The mixture of preservatives was able to inhibit completely the growth of yeast cells, even at the lower concentrations; none of the tested species was able to show visible growth after 21 days, even on the medium with 100 ppm of potassium sorbate and 50 ppm sodium benzoate.

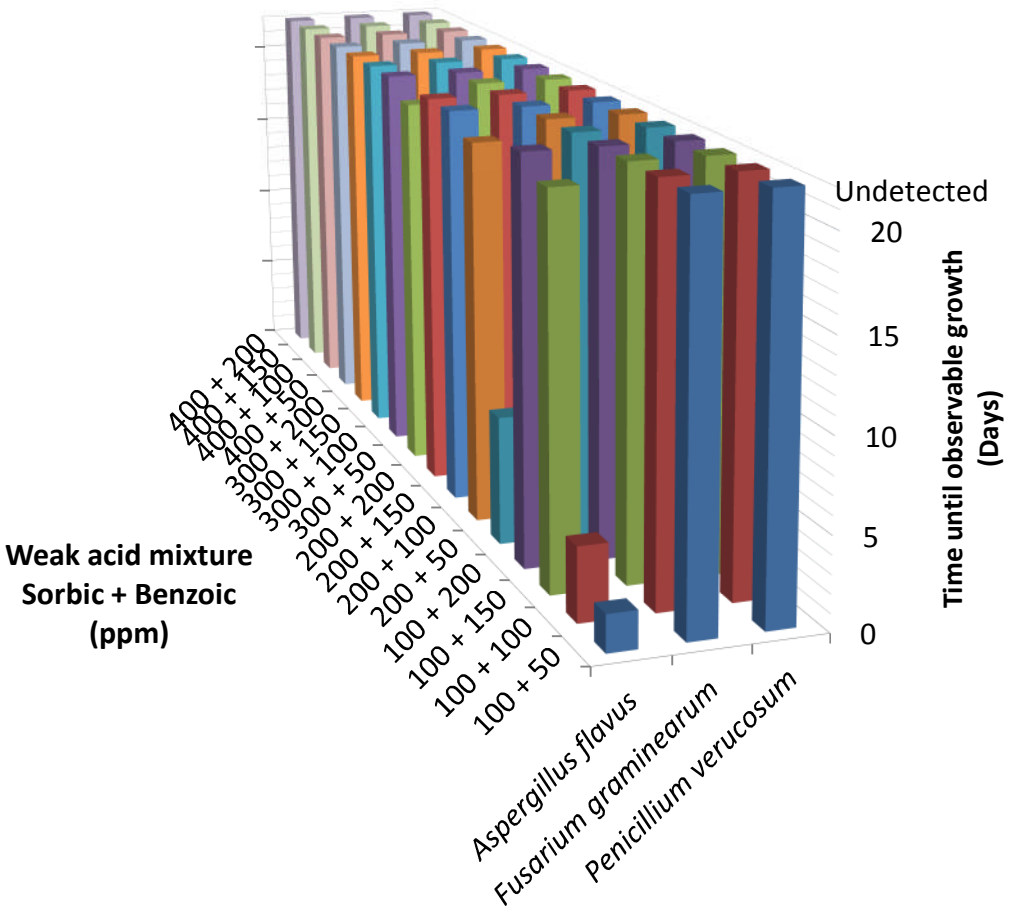

Figure 2.8 - Effect of mixture of potassium sorbate and sodium benzoate on the time to visible growth of mycotoxigenic filamentous fungi.

The use of alternative weak organic acids, as substitutes of benzoate, was not as effective inhibiting growth. The yeast species *Pichia membranifaciens* and *Zygosaccharomyces bailii* had detectable growth when these alternative weak acids were used (Figure 2.9 and 2.10).

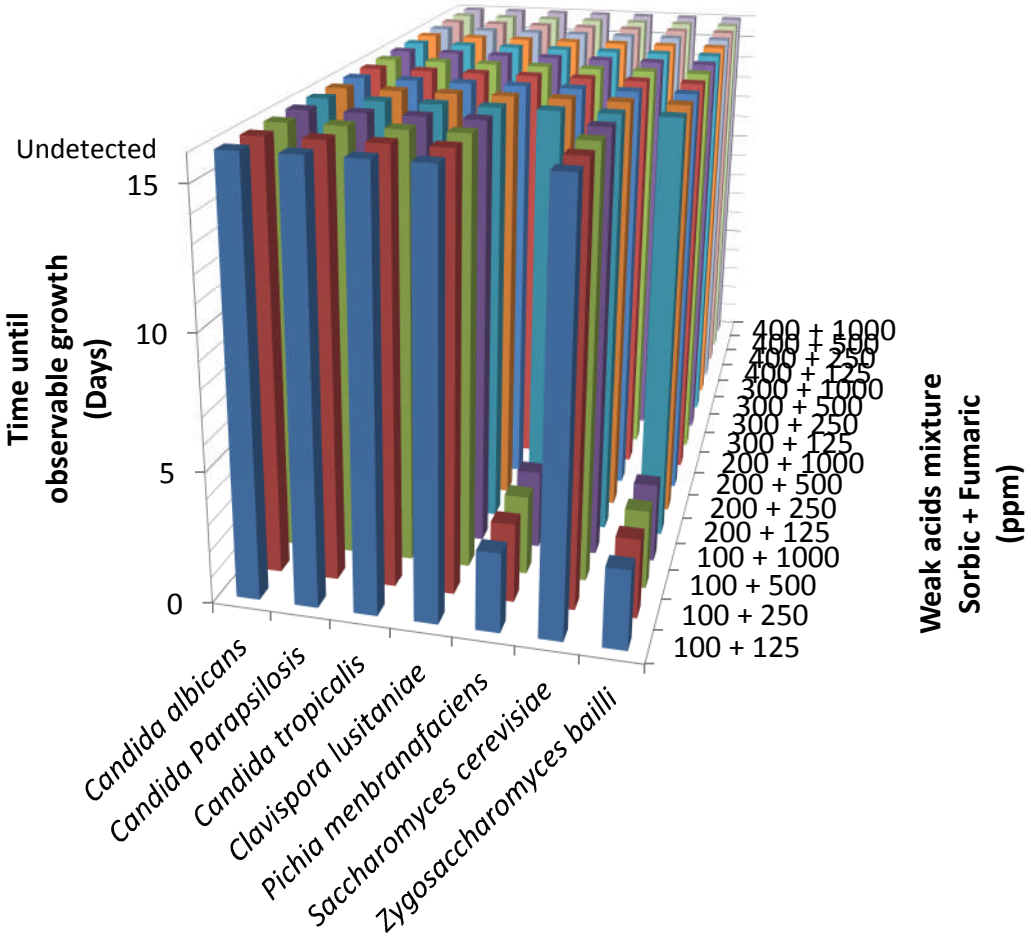

Figure 2.9 - Effect of mixture of potassium sorbate and fumaric acid on the time to visible growth of all yeast species.

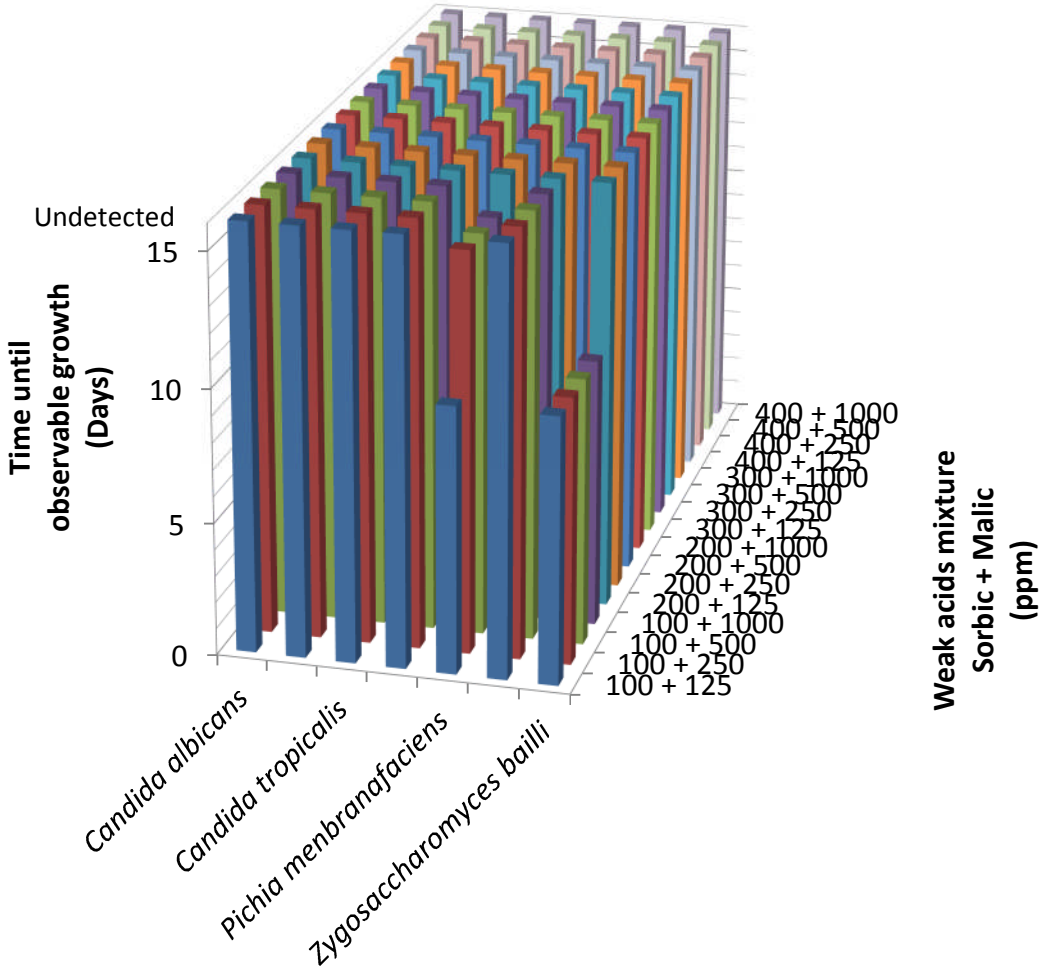

Figure 2.10 - Effect of mixture of potassium sorbate and malic acid on the time to visible growth of all yeast species.

Furthermore, *P. echinulatum, P. glabrum* and *P. spinulosum* were able to grow at all concentrations of fumaric acid mixtures with 100 ppm of potassium sorbate while in the presence of sodium benzoate they were only able to do so at the lower concentrations of benzoate or their growth was completely inhibited. Moreover also *A. puniceus* displayed growth in the presence of malic acid while the same was not observable in the presence of mixtures of benzoate and sorbate (Figure 2.11 and 2.12).

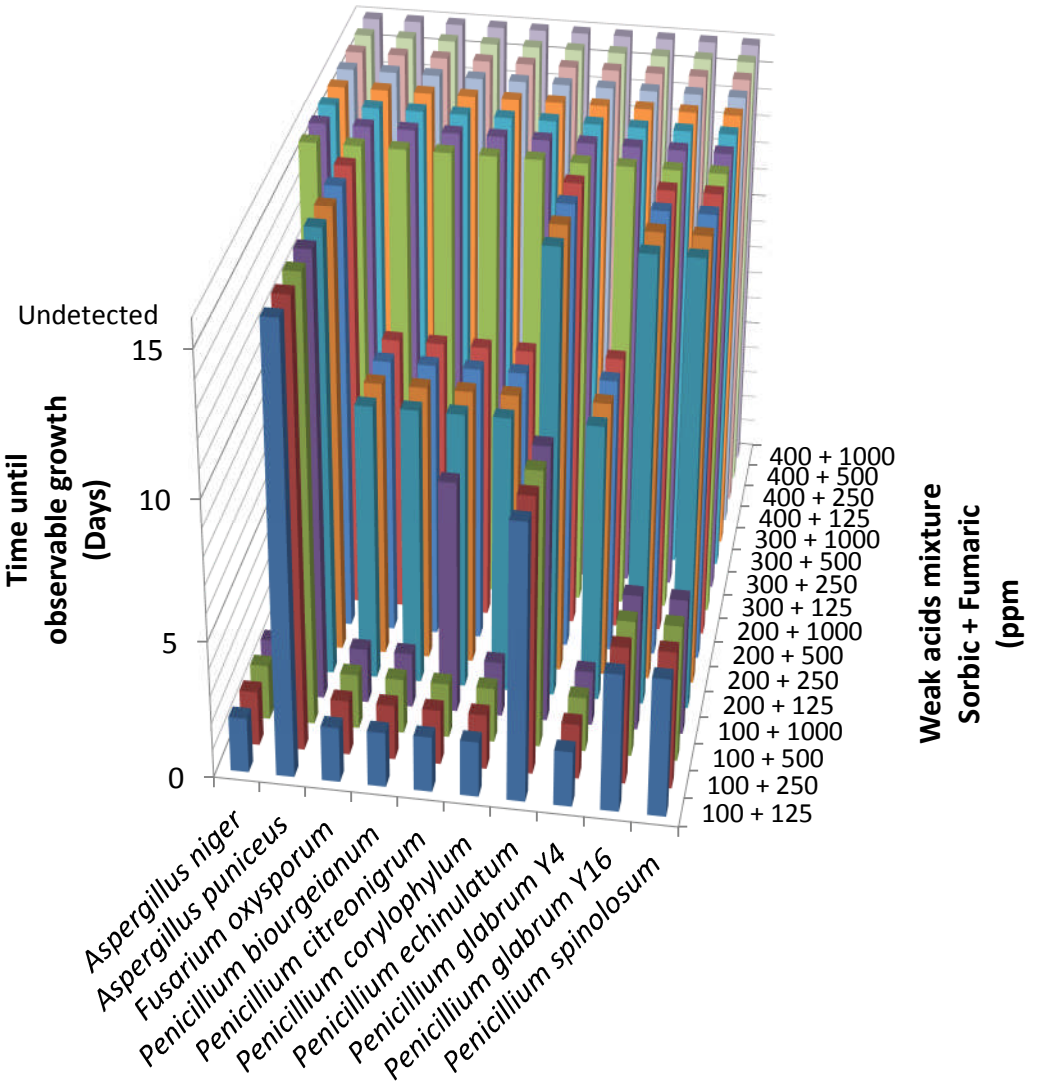

Figure 2.11 - Effect of mixture of potassium sorbate and fumaric acid on the time to visible growth of filamentous fungi.

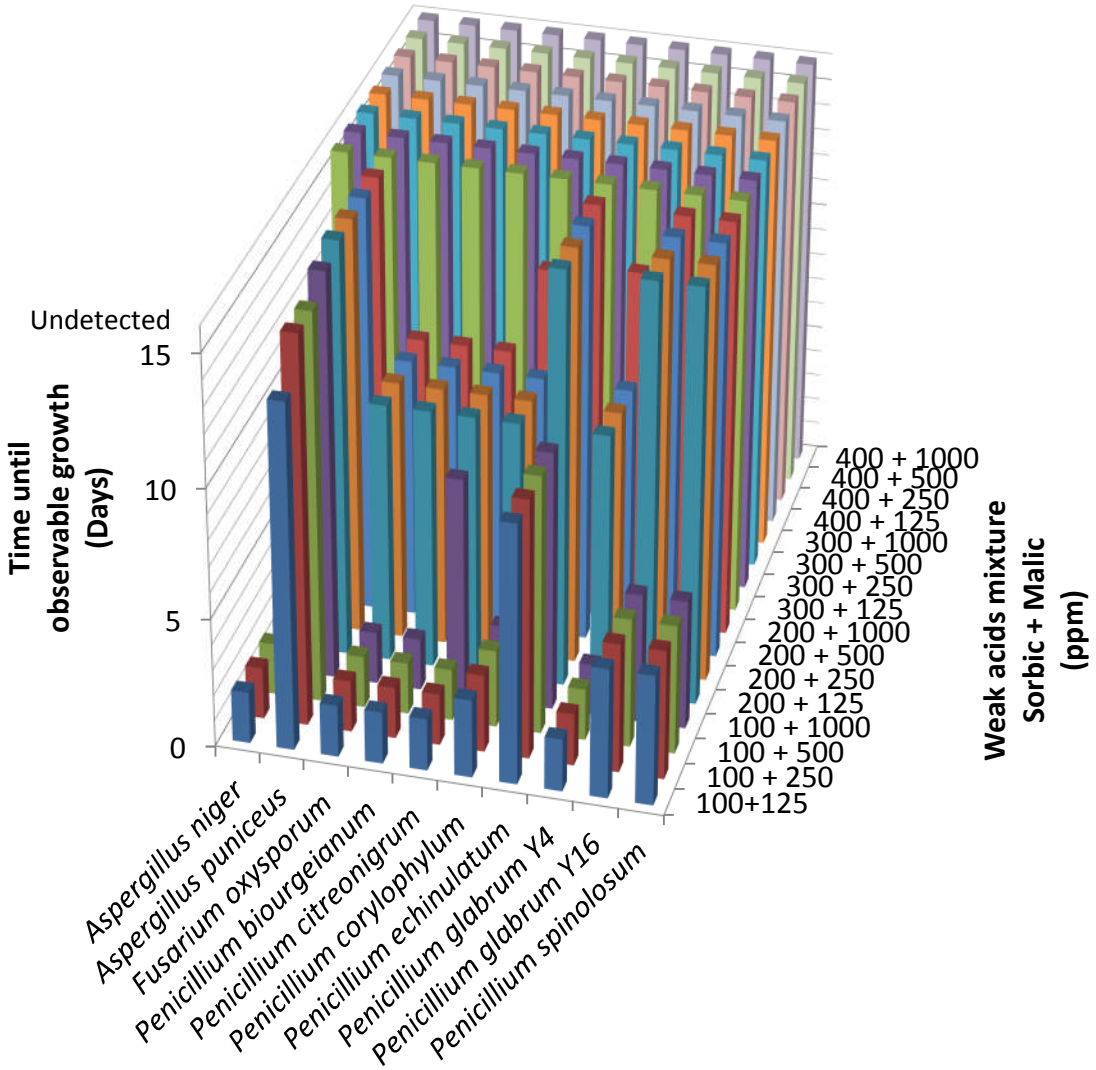

Figure 2.12 - Effect of mixture of potassium sorbate and malic acid on the time to visible growth of filamentous fungi.

The growth of a mycotoxigenic strain of *A. flavus* was in fact stimulated by the presence of fumaric and malic acid. *A. flavus* displayed visible growth at all concentrations of fumaric and malic acid mixtures after 5 days, Figure 2.13 and Figure 2.14 respectively. Detailed data in Appendix A3.

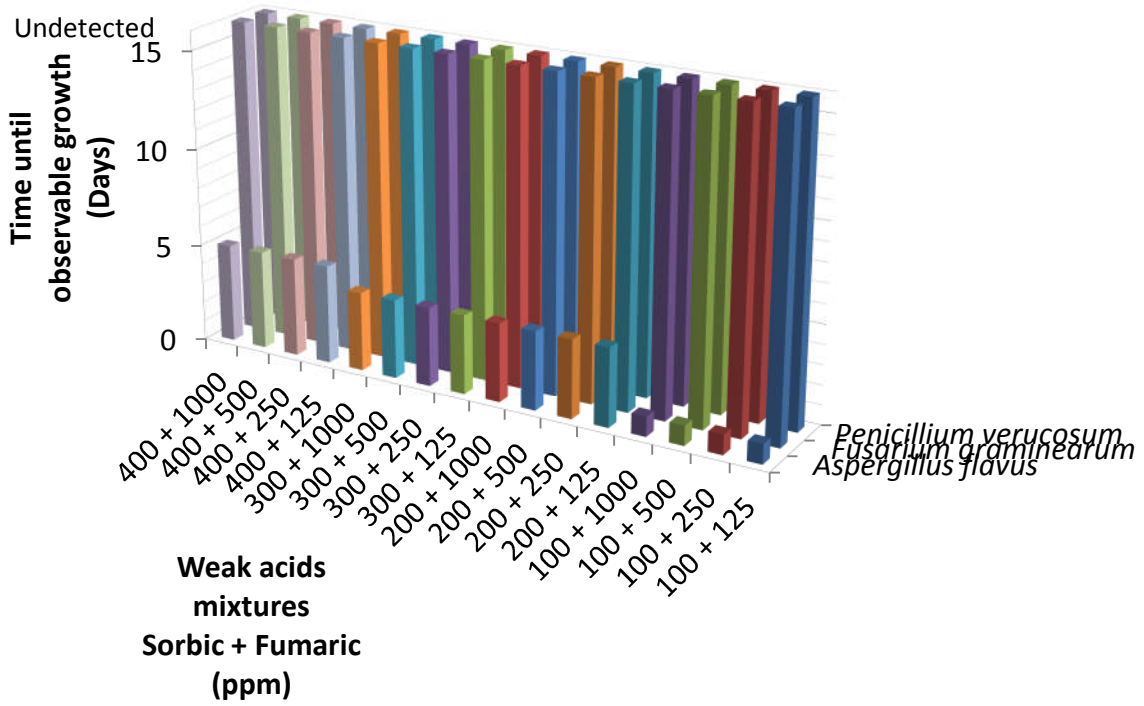

Figure 2.13 - Effect of mixture of potassium sorbate and fumaric acid on the time to visible growth of mycotoxigenic filamentous fungi.

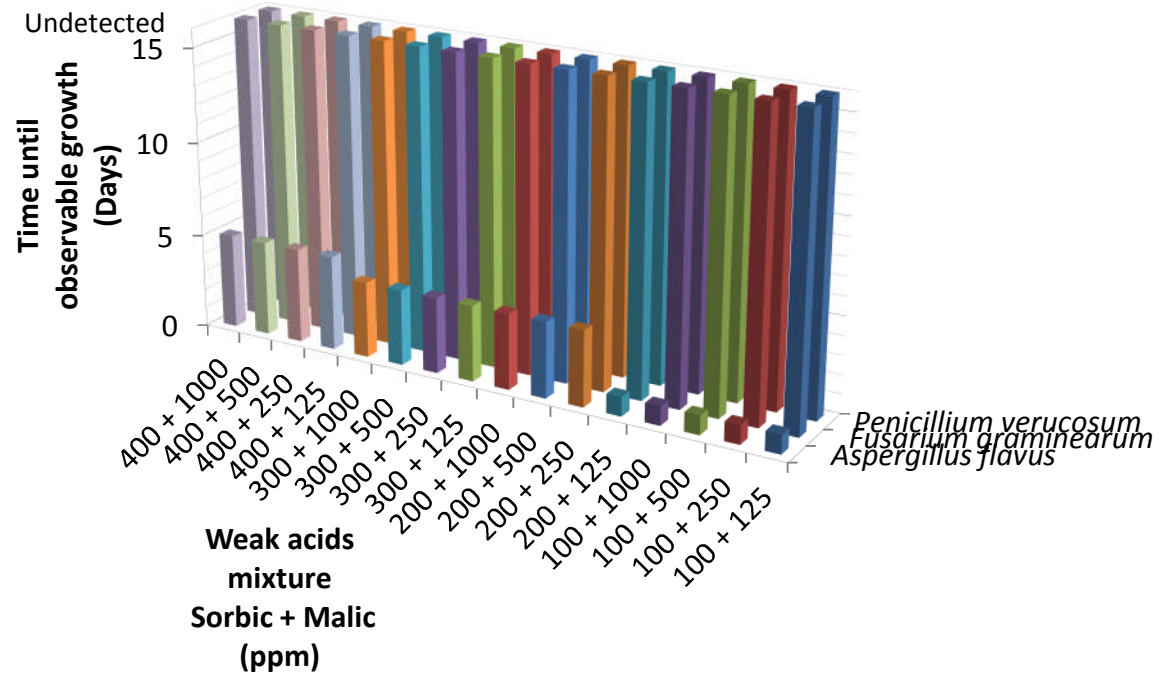

Figure 2.14 - Effect of mixture of potassium sorbate and malic acid on the time to visible growth of mycotoxigenic filamentous fungi.

2.4 Discussion

2.4.1 pH effects on the growth of spoilage fungi

The liquid media based study of how the pH and the method for adjusting the pH, affects growth of spoilage fungi, was very useful in providing the platform to build upon. The results obtained were, in general, similar for all species of filamentous fungi tested (*Aspergillus flavus, Aspergillus niger, Aspergillus puniceus, Fusarium graminearum, Fusarium oxysporum, Penicillium biourgeianum, Penicillium citreonigrum, Penicillium corylophilum, Penicillium echinulatum, Penicillium glabrum, Penicillium spinulosum* and *Penicillium verrucosum*) and they confirmed the known ability for filamentous fungi to withstand low pH conditions, and with the exception of the yeast *Saccharomyces cerevisiae* all other spoilage yeasts were also capable of growing at the lower pH conditions (*Candida albicans, Candida parapsilosis, Candida tropicalis, Clavispora lusitaniae, Pichia membranifaciens* and *Zygosaccharomyces bailii*).

The observation of how the weak acid buffered pH media affected several species of yeasts and filamentous fungi is something to consider especially if work like this are carried out with the optimal goal of creating a predictable model for the growth of spoilage fungi on foodstuff. This could be due to the buffering capacity of the KH-Phthalate. Some studies suggest that this is mainly due to the dissociated form of phthalic acid used to create the buffer, and that it falls into the normal method of action of weak acids (Eklund, 1983). Due to the limited shift in the un-buffered liquid media after 15 days growth of spoilage fungi, this suggests that this was indeed the effect of the dissociated form of the weak acid, acidifying the cytoplasm and inhibiting glycolysis. Nevertheless, and most important result is the knowledge of how the different species of spoilage fungi reacted to lowered pH on defined nutrient-limited liquid media.

2.4.2 Effect of single preservative/weak organic acid solutions on growth of spoilage fungi

This study was very important, since it showed that single preservative use and low pH levels are not sufficient to inhibit growth of spoilage fungi. Filamentous fungi were especially resistant to the single preservative used. But even the yeast species tested were capable of coping with the higher concentrations, and above the legal limit, of potassium sorbate (300 ppm) and sodium benzoate (150 ppm). With only *Candida albicans* requiring five to seven days to display observable growth, in the higher concentrations of potassium sorbate (400 ppm) and sodium benzoate (200 ppm). If we consider that the KH-phthalate effect on the pH study was also due to its activity as a weak acid, then even though the total amount used was very high (2000 ppm) at pH 3 it was also capable of partially inhibiting *Candida albicans* and *Candida tropicalis* and most surprising *Penicillium verrucosum* which was the only filamentous fungi that did not display growth at pH 3 after 20 days incubation at 25ºC. Phthalic acid is not an authorized food additive, is main industrial applications are on the manufacture of dyes, perfumes, pharmaceuticals, and synthetic fibres, as so it should not be consider for this study.

An interesting result was the differences observed between strains of *Penicillium glabrum* (Y4 and Y16) and *P. glabrum* Y16 with the latter needing on average 3 to 5 days longer to grow at the same level as *P. glabrum* Y4. These results were consistent throughout all the growth boundary studies, and may imply differences in the resistance mechanism between both strains. This fact is of particular importance if we consider the construction of predictive models of spoilage fungi and it might explain why different authors show models with different levels of tolerance by the same species (Battey *et al.*, 2001; Battey *et al.*, 2002; Guynot *et al.*, 2004; Parra and Magan, 2004). With this in mind it would be advisable to execute model validation using different strains, from those responsible for the model construction in the first place, and gathering data from several strains of the same species (Parra and Magan, 2004). Some might argue that the effect might have been the result of adaptation to the medium, since the cultures were sub-cultured on Malt Extract based media many

times. For this reason all of the spoilage fungal species used were recovered from the original frozen aliquots at the same time, and only used after three sub-cultures on MEA. Moreover at the time of each spore/cells suspensions were made, all cultures had been sub-cultured in a similar way. This suggests that the results are indeed consistent.

2.4.3 Effect of mixtures of preservative/weak organic acid solutions on growth of spoilage fungi

The combined effect of mixtures of weak organic acids is the reason behind their wide application on the beverage and food industries; this does not however mean that their effect has been properly examined against spoilage fungi. For this reason, matrices of varying concentrations of weak organic acids were tested against all 20 species of spoilage fungi.

Even though most of the data collected supports the "Gamma Hypothesis", that the observed inhibition is nothing more than a complex additive effect of each of the individual weak organic acids effects, there are some exceptions to this hypothesis. The most apparent case of synergism is the effect of potassium sorbate and sodium benzoate had on inhibiting yeast growth. Individually none of them was capable of inhibiting growth of any of the yeasts species for more than 7 days, even at concentrations well over 150 ppm, while together just 100 ppm of sorbate and 50 ppm of benzoate was sufficient to inhibit growth of all yeasts species for up to 21 days. On the other hand the inhibition effect of these weak organic acids, when considering just the filamentous fungal species it might suggest an additive effect confluent with the gamma hypothesis. However, even just considering the filamentous fungi we can also verify some synergism between preservatives. For instance, all filamentous moulds were capable of producing observable growth after 5 days on media containing 400 ppm of potassium sorbate, but the mixture of 300 ppm of sorbate with 50 or 100 ppm of benzoate, or 200 ppm of benzoate with 150 or 200 ppm was capable of inhibiting growth of all filamentous fungal species, with the exception of *Aspergillus niger*, but even this species only displayed growth after 7 days. If we analyse the effect of

mixtures on individually then the additive effect appears to be clear. It is more difficult to confirm the synergistic effects generally.

These results can be partially explained by the different mechanism of action that is pointed out for sorbic acid and its salts, like potassium sorbate. It is believed that sorbic acid, unlike the other weak organic acids, has a membrane active compound role (Stratford and Anslow, 1998), this interference with membrane permeability might be the reason behind this apparent synergetic effect between this preservative and other weak organic acids.

The use of fumaric and malic acid produced good overall inhibition, considering these weak organic acids are considered natural additives by the FDA, and malic acid in particular has GRAS status, and both can be used at high concentrations.

The main conclusion from the mixture of potassium sorbate and malic or fumaric acid, is that the observable growth appears to be more related with the ability of sorbate to interfere with the membrane than the capacity of these weak organic acids to inhibit growth. These results suggest that the capacity of potassium sorbate to interfere with membrane proteins might facilitate the transport of the dissociated forms of the acids into the cytoplasm, where they would be responsible for acidifying the cytoplasmatic pH, inhibiting the glycolysis enzymes and consequently growth. These would explain why only on media with mixtures of these weak organic acids, with up to 200 ppm of sorbate it was possible to observe growth, irrelevant of how high the concentration of malic or fumaric acid used in the mixture. For example, there was more observable growth in a mixture of 100 ppm of sorbate with 1000 ppm of either weak organic acid, than in a mixture containing 300 ppm sorbate and 125 ppm of either acid. It was more relevant to note that the growth of the first mixture is similar to that observed in a mixture of 100 ppm of sorbate with 125 ppm of either acid. There were some differences between replicates, with higher concentrations (1000 ppm) of malic or fumaric acid mixed with 100 and 200 ppm of potassium sorbate suggesting that at this level, the amount of fumaric and malic acid might start to influence the inhibition of observable growth of the spoilage fungi.

Nevertheless the use of these alternative weak acids also stimulated the growth of *A. flavus* an important mycotoxigenic fungus. *P. verrucosum* and *F. graminearum* were not able to grow in the presence of mixtures of these weak acids. Previously *P. verrucosum* showed capacity to grow even in the presence of above the legal limit amounts of sodium benzoate and potassium sorbate. In fact, from the mycotoxigenic species only *F. graminearum* had the growth inhibited by the presence of legal amounts of the preservatives studied. Nevertheless these results suggest an increased risk of mycotoxin contamination at these low nutrient conditions typical of final food chain and industrial environments.

3 Rapid High throughput RNA extraction method development

3.1 Introduction

Rapid advances in molecular biology have promoted the use of molecular techniques in mycological studies. As an example, new research trends with mycotoxigenic fungi have integrated traditional ecological and physiological experiments with molecular data (gene expression and gene switching) to use a system approach to try and provide better insights into the functional role of gene clusters involved in mycotoxin production and a better understanding of fungal behaviour, especially in relation to environmental stresses (Schmidt-Heydt *et al.*, 2009; Schmidt-Heydt *et al.*, 2010b; Abdel-Hadi *et al.*, 2011; Schmidt-Heydt *et al.*, 2011).

Thus, the adequate isolation and purification of fungal RNA is a critical step to ensure the successful application of these techniques. The structure of the fungal cell wall makes their disruption for nucleic acid extraction difficult. For this reason the use of standard RNA extraction methods developed for animal cells, bacteria or yeasts cannot be readily applied.

The most common techniques for fungal nucleic acid extraction are the use of cell wall degrading enzymes (Einsele *et al.*, 1997; Williamson *et al.*, 2000) and a physical grinding treatment undertaken with either dry ice or liquid nitrogen (Al-Samarrai and Schmid, 2002; Griffin *et al.*, 2002; Loeffler *et al.*, 2001). For RNA extraction this last physical treatment is the most common methodology applied. It consists of freezing the mycelium with liquid nitrogen and grinding it with the aid of a mortar and a pestle (Abdel-Hadi *et al.*, 2010; Schmidt-Heydt *et al.*, 2007).

A number of studies have reported promising results for mechanical bead-beating extraction procedures to isolate fungal DNA (de Boer *et al.*, 2010; Griffiths *et al.*, 2006; van Burik *et al.*, 1998;), but no special attention has been paid to methods for the isolation of RNA from cultured mycelium, either in liquid or solid agar media. The technique has several advantages over the traditional methods including reduction in time, multiple sample extraction and a reduction of the risk of cross contamination.

There is also no need for liquid nitrogen, which is often problematic and hazardous, especially when a large number of samples need to be processed.

To our knowledge, there is no protocol for RNA extraction using any high speed bead-beating method. The aim of this work was to design and develop a bead beating protocol for the extraction of high-quality RNA samples from filamentous fungal biomass. Thus different glass, zirconium oxide, tungsten carbide and stainless steel beads of various sizes were examined in the Precellys 24 homogenizer (Bertin Technologies, Montigny le Bretonneux, France) and in the TissueLyser LT (Qiagen, UK) instruments. Subsequently, the results obtained were compared with those obtained using the traditional method.

The successful development of this protocol allowed the extraction of multiple samples at different conditions aiding the RT-qPCR studies of key mycotoxigenic genes, namely *Tri5* and *otapks*Pv of *Fusarium graminearum* and *Penicillium verrucosum.* Furthermore, it opens the prospect of using high throughput growth methods permitting the use of low amounts of mycelium for successful extraction of quality RNA. *Aspergillus flavus* was used due to previous RT-qPCR work by Abdel-Hadi *et al.* (2010) using this filamentous fungus allowing faster development and validation of the method.

3.2 Materials and methods

3.2.1 Fungal species and isolates maintenance

An *Aspergillus flavus* strain (NRRL 3357) was used in this study. The strain was maintained on Malt Extract Agar (MEA, Oxoid, UK) at 25ºC. The cultures were subculture on Yeast Extract Sucrose agar (YES) which is composed of 20 g/L of yeast extract (Fluka, USA), 150 g/L of sucrose (Fisher Scientific, UK) and 15 g/L agar (Oxoid, UK). To enable fungal biomass harvesting, a sterile cellophane overlay disc (8.5 cm) was placed aseptically on the surface of each 9 cm Petri plate prior to inoculation.

3.2.2 Inoculation, incubation and mycelium collection

Spores from a 7 day culture were collected using a sterile loop and placed in a sterile 25 mL Universal bottle containing 10 mL of reverse osmosis (RO) water (Direct-Q 3 UV, Millipore, France) with 0.05% Tween 80. The spore suspensions was counted using a haemocytometer and adjusted to $1x10^5$ spores per mL. Petri plates with YES and cellophane overlays were centrally inoculated with 10 µL of the spore suspension and incubated at 25ºC during 10 days. After the incubation period, the cellophane discs containing whole colonies were collected under sterile conditions, quickly frozen in liquid nitrogen and stored at -80ºC until RNA extraction.

3.2.3 RNA extraction methods

In order to improve the extraction methodology and to achieve a high efficiency and reduced variability in the total RNA yields, several automated and traditional methods were compared.

In the interest of ensuring that the RNA samples obtained were suitable for RT-qPCR experiments, the MIQE guidelines (Bustin *et al.*, 2009) were followed. The samples were quantified and the absorbance ratios including the A_{260}/A_{280} ratio were measured using the Picodrop® (Picodrop Limited, Saffron Walden, UK). Gel electrophoresis, and RNA quality indicator numbers (RQI) were obtained using the Experion™ system (Bio-Rad Laboratories Ltd., Hertfordshire, UK).

(a) Mortar and pestle method

The traditional method of grinding the mycelium in a mortar with a pestle using liquid nitrogen is the most common technique to extract RNA samples from filamentous fungi. Thus a sample of between 0.65 - 1 g biomass was ground in liquid nitrogen to a fine powder using the mortar and pestle. 150 mg of this powder was then transferred to a 2 mL Safe-Lock tube (Eppendorf, Germany), and 750 µL of TRIzol® (Invitrogen, USA) or RLT buffer (Qiagen, UK) were added. After a quick vortex (10 seconds) the samples were frozen at -80ºC until RNA purification. TRIzol® was initially used due to its recognised RNA stabilizer properties, later the RLT buffer supplemented with 10 ppm

of β-mercaptoethanol was used so that an evaluation between both extraction buffers could be achieved.

(b) Bead beating method

A sample of 150 mg of frozen biomass was placed into a 2 mL extraction tube containing beads. 750 µL of TRIzol®, which denatures proteins and inhibits endogenous RNase activity and thus improves RNA stability while still facilitating cell wall disruption, was added. The lysing properties of the RLT buffer (provided with the RNeasy® Plant Mini Kit) (Qiagen, Germany) were also evaluated; TRIzol® was replaced with the same amount of RLT buffer supplemented with β-mercaptoethanol. After a quick vortex the tubes were frozen at -80ºC until required.

Table 3.1 - Summary of the beads characteristics including: reference, material and size used in these studies.

Reference	Material	Size (mm)
VK 01	Glass	0.1
VK 05		0.5
CK 14	Zirconium Oxide	1.4
CK 28		2.8
TC 3	Tungsten Carbide	3.0
SS 5	Stainless Steel	5.0
SS 7		7.0

The extraction was carried out in two different bead beaters, the TissueLyser LT (Qiagen, UK) which has a characteristic 2D vertical movement with up to 50 oscillations per second, and the Precellys 24 (Bertin Technologies, Montigny le Bretonneux, France) with 3D motion with up to 6500 RPM. The bead material evaluated included glass, zirconium oxide, tungsten carbide and stainless steel beads of various sizes (Table 3.1). In conjunction with the TissueLyser LT, all beads supplied by the manufacture were tested, even though smaller size beads are recommended for use

with filamentous fungi. In the Precellys we also tried all available beads sizes, although we did not try stainless steel beads since zirconium oxide beads were hard enough to break the mycelium cell wall and the 2.8 mm sized beads could be compared to the 3 mm tungsten carbide beads since both are ceramic materials. Both systems were used at their maximum speed. Two types of procedure were used:

Stainless steel and tungsten carbide beads were used in conjunction with the TissueLyser LT, with all samples agitated for 1 minute with a 10 second interval followed by another 1 minute agitation. During the interval and after the cycle, the samples were cooled down by placing them in ice.

The glass and zirconium oxide beads were used with the Precellys 24. They were agitated for 25 seconds followed by a 5 second interval and another 25 seconds agitation. Afterwards samples were immediately cooled down by placing them in ice.

In both cases, the final mixture was centrifuged at 16000 g for 10 min at 4ºC in a temperature controlled centrifuge. The supernatants were collected in a 2 mL Safe-Lock tube (Eppendorf, Germany).

(c) Bead beating parameters optimization

Different cycles were assessed in order to increase the total RNA extraction. Modifications consisted of increasing the beating time by up to 5 minutes, increasing the cooling in between the cycles for up to 5 minutes and increasing the beating cycles and adding cooling steps.

3.2.4 Evaluation of variability and repeatability

The precision was determined by repeatability (intra-day) and intermediate precision (inter-day). Repeatability was evaluated by assaying 6 samples (3 + 3) from the same biomass sample on the same day. Intermediate precision was studied by comparing 12 samples (6 + 6) on different days. The intra-operator variability was evaluated by independent analyses of 12 replicates of the same sample on the same day by two different operators. Total RNA concentration relative standard deviations (RDS) were calculated.

3.2.5 Total RNA purification and integrity evaluation

The purification was carried out using the QIAcube® (Qiagen, Germany), using the RNeasy® Plant Mini Kit (Qiagen, Germany), with the appropriate protocol. The RNA concentration was determined spectrophotometrically using a 2.5 µL aliquot on the Picodrop™, the A_{260}/A_{280} ratio was also obtained for initial RNA purity assessment. To fully evaluate RNA integrity and quality, 1 µL aliquot was used as described by the manufacturer on the Experion™ system using the RNA StdSens Analysis chip (Bio-Rad, UK). An electropherogram was generated by the Experion™ system for each sample from which the RNA Quality Indicator (RQI) was automatically calculated and a virtual gel could be generated.

3.2.6 Statistical analysis

Analyses were done using the JMP 8 Statistical Software package (SAS Institute Cary NC, USA). Generally, the independent variable distribution was assessed using the Shapiro-Wilk W Test and the homoscedasticity was tested using the Levene's test. When any of them failed, a variable transformation was performed in order to improve normality or homogenize the variances. Either non-parametric (Kruskal-Wallis rank sum test) or parametric ANOVA test were used depending on the dataset characteristics. Afterwards *post-hoc* Tukey-Kramer Honestly Significant Difference (HSD) test was applied to investigate the relationship between the different treatment averages (Abdi and Williams, 2010).

3.3 Results

3.3.1 Comparison between the traditional and bead-beating methods

An overall comparison of total RNA obtained from each 100 mg of biomass extracted, for all methods tested, is shown in Figure 3.1. Data was analysed using the Kruskal-Wallis test and significant differences were found between the total RNA amounts isolated using the different bead treatments and the manual system as factors (*p-value*=0.0072). Further analysis using the Tukey-Kramer HSD test revealed that the total RNA obtained using the manual method was statistically lower than the total RNA

obtained using glass and zirconium beads (VK01, VK05, CK14 and CK28). Consequently, stainless steel and tungsten carbide beads were removed from the data set.

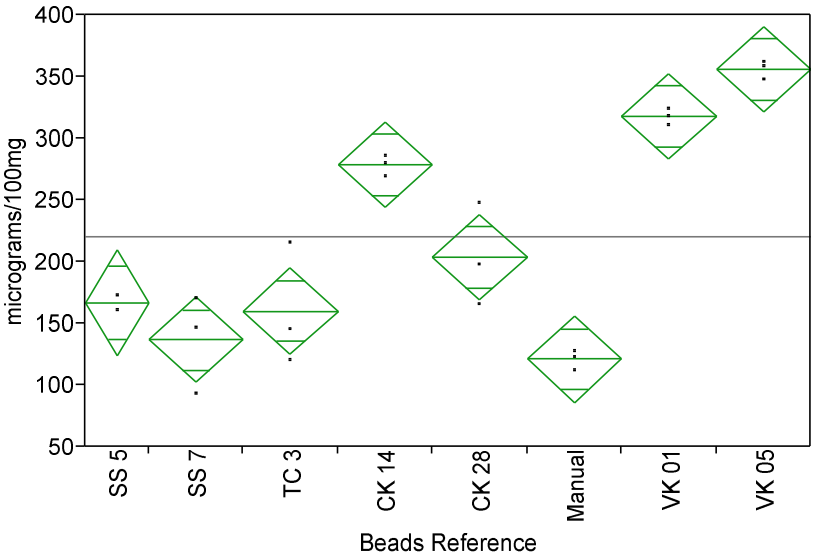

Figure 3.1 - Diamond diagram obtained during Kruskal-Wallis test analysis of OD values/100mg of biomass using the different beads as factors (p-value=0.0072). The line across each diamond represents the group mean. The vertical span of each diamond represents the 95% confidence interval for each group. Key to treatments: SS - Stainless Steel, TC – Tungsten Carbide, CK – Zirconium Oxide, VK – Glass; followed by the bead size code 01 – 0.1 mm, 05 – 0.5 mm, 14 – 1.4 mm, 28 – 2.8 mm, 3 – 3.0 mm, 5 – 5.0 mm and 7 – 7.0 mm.

The remaining data (including glass and zirconium beads and the manual method) were analysed by ANOVA. Results confirmed the statistically significant difference in the total RNA obtained with the different procedures (p-value<0.0001). Additional *post-hoc* analysis confirmed that glass beads provided the best recovery yields. Among them VK05 (glass beads of 0.5mm diameter) resulted in the highest amount of total RNA. Statistical analysis did not show any differences with VK01 but differences were found with respect to all the other treatments. Results of the average total RNA extracted per 100 mg of biomass are shown in Figure 3.2.

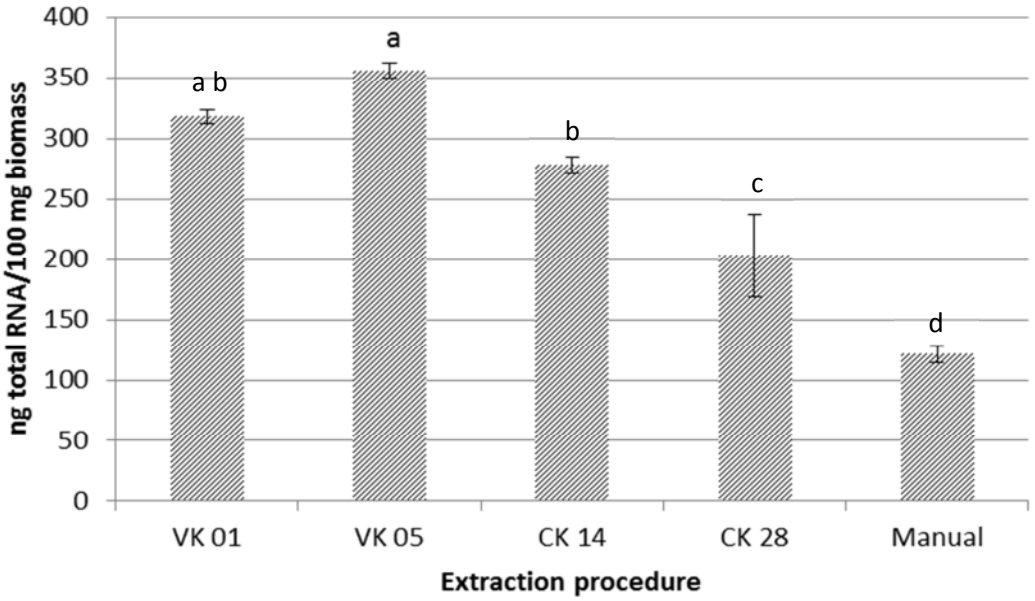

Figure 3.2 - Total RNA yield average per 100 mg of initial biomass and standard deviation comparing different beads with the manual method. Groups connected by different letters represent statistically different groups using Tukey-Kramer HSD test (p-value<0.0001). Key to treatments: CK – Zirconium Oxide, VK – Glass; followed by the bead size code 01 – 0.1 mm, 05 – 0.5 mm, 14 – 1.4 mm, 28 – 2.8 mm.

In order to verify the general quality of the RNA, the A_{260}/A_{280} ratio was measured in all the samples. Measurements were performed in water under neutral pH conditions using the Picodrop. Ratios obtained for the manual method as well as for the bead beating method were close to the threshold number of 2 and were considered satisfactory. Based on the overall results obtained and based on the higher total RNA yield, optimum absorbance ratios and low variability of the results, the VK05 glass beads were selected as the best for extraction of filamentous fungal RNA.

3.3.2 Glass bead beating protocol optimization and quality parameters

Further modifications were examined with the objective of increasing the total RNA extraction yield using the VK05 glass beads. In order to discriminate between treatments, different quality parameters and thresholds were used. As a first threshold of quality, trials in which the A_{260}/A_{280} ratio were not in the range between 1.8 and 2.1 were rejected. It should be noted that the absorbance ratios only provides an indication of the RNA purity, so in order to ensure that the RNA obtained using the different bead beating protocols had conserved integrity and could be used for

quantitative analysis, the Experion™ system was used to study the total RNA quality and integrity. As an example of our quality threshold a virtual gel generated from the electropherogram of samples extracted using different glass bead beating protocols, including 2 rejected and 2 accepted samples, are shown in Figure 3.3. Lane's 2-3 show admissible absorbance ratios but which failed our integrity threshold. This shows that while a satisfactory amount of RNA was present, degradation was evident. This is reflected in the RQI numbers which were <4.5. Samples that showed un-denatured RNA, Lane 1, were analysed again after an extended denaturation step. Samples that displayed genomic DNA contamination, Lane 5, were accepted even though their use in RT-qPCR experiments was subjected to a DNase treatment. Only samples with electropherograms similar to lane 4 were immediately accepted. In Lanes 4-5 good integrity of the two RNA subunits (18S and 28S) can be observed and RQI values were >6.5, a value that was adopted as a good quality threshold. Samples that displayed a RQI between 4.5 and 6.5 would require further analysis if they were to be used in RT-qPCR experiment.

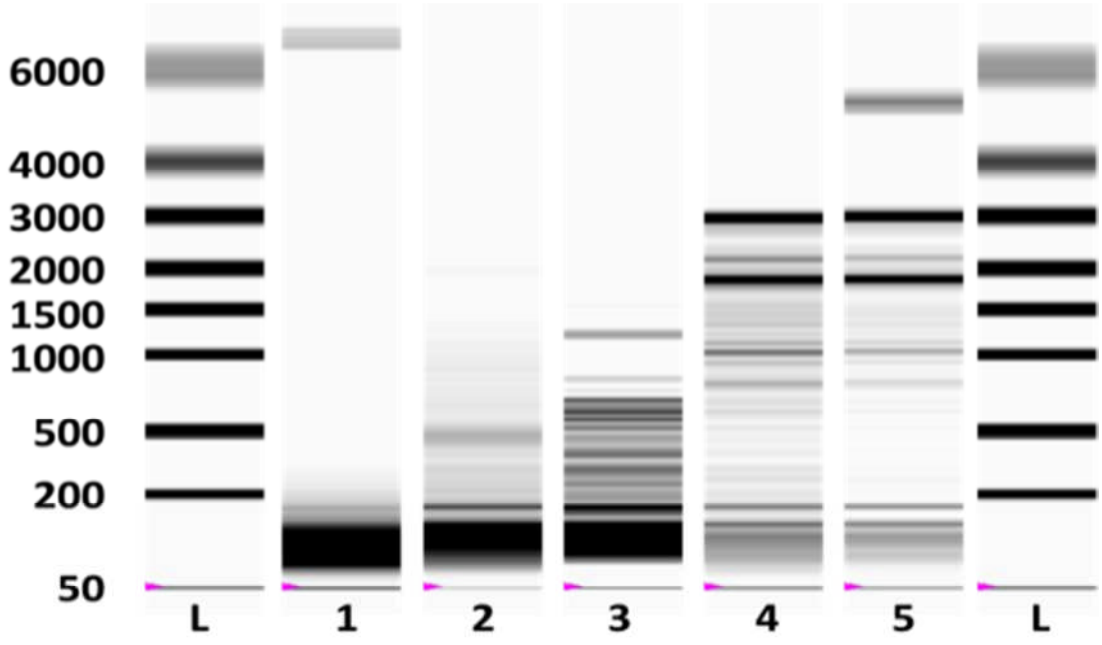

Figure 3.3 - Virtual gel generated by BioRad Experion for samples of different quality (1-5). Lane 1 appears to possess un-denatured RNA, Lane 2 and 3 highly degraded RNA and Lane 4 and 5 have a RQI above 6.5 (Good Quality). Lane 5 shows some genomic DNA contamination.

Final comparisons were made between protocols with VK05 glass beads by extracting 12 replicates of the same fungal biomass sample. Six were extracted using the methodology described in Section 2.3.2b (25 secs + 5 secs cooling + 25 secs) and the other 6 by agitating for 15 secs followed by 5 mins cooling, 25 secs of agitation, followed by another 5 mins of cooling and a final cycle of agitation for 15 secs. The second method gave a total RNA final yield of 20.7% higher than the former. RQI numbers ranged between 6.5 and 9.7 in the shorter protocol described in Section 2.3.2b which included two beating cycles of 25 secs interrupted by a cooling step of 5 secs and from 3.0 to 6.3 in the protocol that included more cooling steps, Figure 3.4 and 3.5. Increasing the beating period lead to a rapid decrease in RNA integrity with most samples having complete degradation of the ribosomal RNA by increasing the beating period to >2 mins.

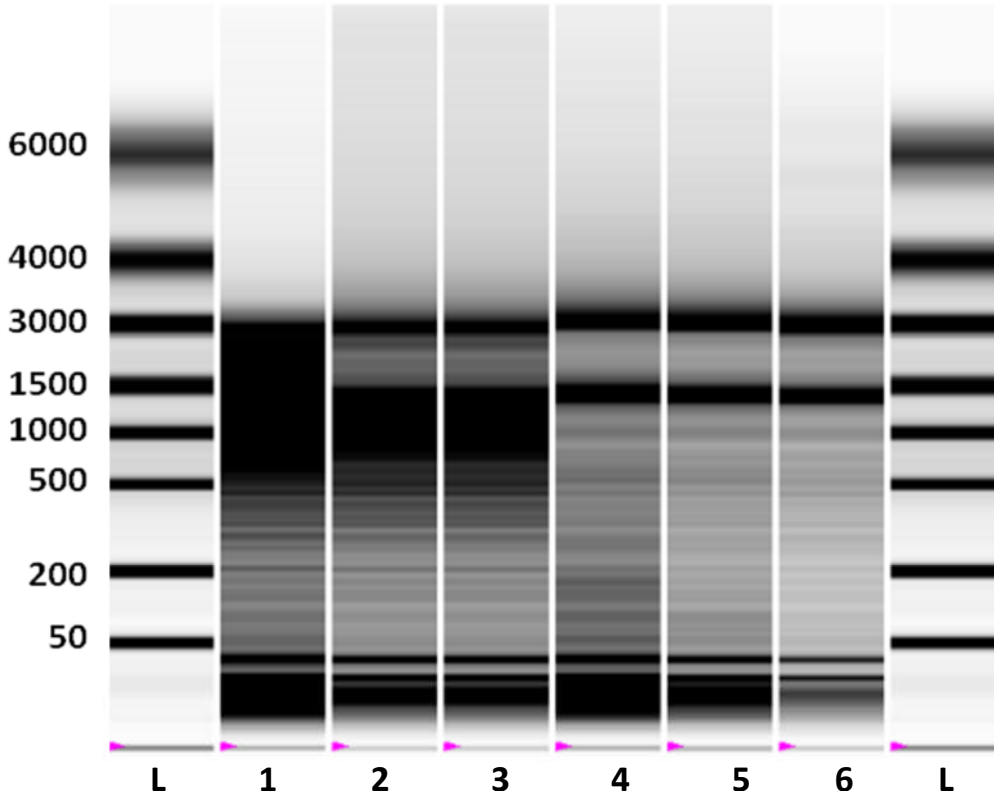

Figure 3.4 - Virtual gel generated by BioRad Experion of samples extracted using different bead beating protocols. Lane 1, 2 and 3 samples were subjected to a 5 minute ice cooling step in between bead beating steps, Lane 4, 5 and 6 samples that were only subjected to a 5 second interval at room temperature between bead beating steps. The RQI of Lane 1 is 3.0, Lane 2 is 3.7, Lane 3 is 4.1, Lane 4 is 6.5, Lane 5 is 6.7 and Lane 6 is 7.7 on a 0-10 scale.

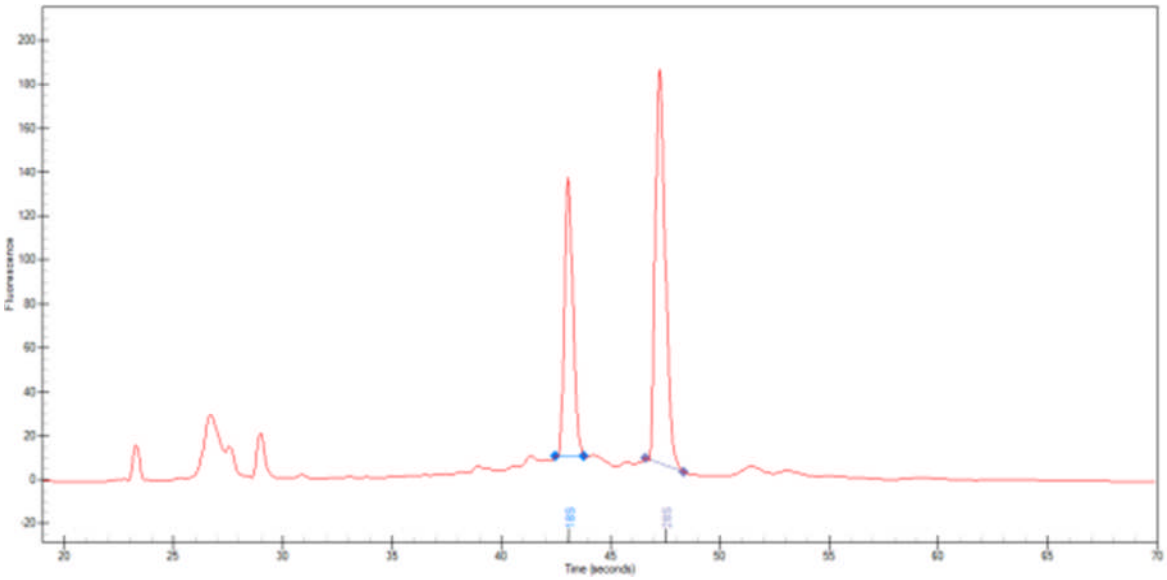

Figure 3.5 - Electropherogram of a good quality RNA sample extracted using glass beads with two 25 seconds beating periods paused by only 5 seconds at room temperature. The RQI of this sample is 9.7.

After comparison of RNA integrity RQI numbers the method described in Section 2.3.2b with only 50 secs total beating time in two different cycles with a 5 sec interval to prevent overheating of the samples was selected as the optimum extraction protocol.

3.3.3 Performance studies of the chosen method

Intra- and inter-day and inter-operator variability were tested for the proposed method. Intra-day (6 replicates) and inter-day variability were 8.01% RSD and 9.24% RSD respectively, suggesting good consistency of the proposed method.

The inter-operator variability after extraction of 12 samples (6 samples each operator) was 6.61% RSD. These data were analysed using the Student's statistical test to examine whether there was statistically significant differences between operators. The p-value was 0.66, showing that no significant differences were found.

3.4 Discussion

The cell wall of filamentous fungi is composed of chitin, (1-3)-β-D-glucan, (1-6)-β-glucan, lipids and peptides which affects the ability to effectively extract DNA and RNA for genetic studies (Francesconi *et al.*, 2008). This has often hindered the development of high throughput systems for the extraction of nucleic acids from filamentous fungi.

In this study, we have demonstrated a fast semi-automated high throughput bead-beating protocol for the extraction and purification of total RNA from filamentous fungi with high yields of total RNA while conserving the overall quality and integrity. We have addressed two major bottlenecks that have been found in the recovery of nucleic acids from filamentous fungi. The first was to effectively gain access to the interior of the cell. This step has a profound impact on the total RNA yield and quality. RNA structure and chemical instability allied with high content of endogenous RNase in filamentous fungi means that temperature and buffering conditions can significantly influence not only yield but also RNA integrity and overall quality.

Previous osmotic pressure methods cannot be readily applied as the cell wall of filamentous fungi impedes lysis (Fredricks *et al.*, 2005). The use of enzymes for cell wall digestion is possible; nevertheless the conditions used for enzymatic digestion of the cell wall could result in rapid degradation of the RNA by endogenous RNase activity. This would also increase the already high level of protein contamination of the sample which would potentially affect the downstream applications. Faster and more aggressive methods have also been used for membrane disruption including sonication (van Burik *et al.*, 1998), microwave radiation (Ferreira and Glass, 1996; Tendulkar *et al.*, 2003) and thermal treatment (Liu *et al.*, 2011).

All these methods would have a detrimental effect on RNA extraction. The thermal susceptibility of RNA would exclude thermal treatment as well as microwave radiation. Our data showed that an increase in temperature, caused by increasing the beating period without adjusting the cooling period, lead to dramatic decreases in RNA integrity and quality. Sonication alone has not proved to be very efficient with DNA and for this reason was excluded from our study (van Burik *et al.*, 1998). Most of these

alternatives have only been used with DNA and there are no studies suggesting their effectiveness and efficiency in extracting RNA from filamentous fungi.

Physical grinding methods using the mortar and pestle or bead beating have been recommended to extract DNA, with the latter giving the best extraction efficiency with fungal samples (Haugland *et al.*, 2002; van Burik *et al.*, 1998; White *et al.*, 2010). There are also some method variations which include not only physical grinding methods using the mortar and pestle with sonication (Schmidt-Heydt *et al.*, 2009*)*, but these methods are still very time consuming and still have disadvantages.

Although, recently several authors have demonstrated improved methods to extract nucleic acids, mainly DNA, for PCR screening of clinically important yeasts and moulds (AlShahni *et al.*, 2009; Fredlund *et al.*, 2008; Liu *et al.*, 2011; Manzanilla-López *et al.*, 2009; Zhang *et al.*, 2010), the challenge for a fast high-throughput and reliable protocol that could be widely used in fungi has still not been achieved. Although some faster and easier methods have recently been developed (Liu *et al.*, 2011), these have not been appropriate for more sensitive techniques such as qPCR, where better quality extracted nucleic acids are required (Bustin *et al.*, 2009), and have often been found to be unsuitable, due to the potential degradation of RNA during the initial cell wall disruption.

In the present study, the use of bead beating methods gave consistently high yields of good quality total RNA even though the high speed employed generates a temperature increase during the bead beating process which potentially could also lead to RNA degradation. To assess the influence of cooling steps would have on the RNA integrity, samples were cooled in between each bead beating step for up to 5 minute intervals. During this interval the samples were cooled by placing them in ice or briefly in liquid nitrogen or even by stopping the bead beating and resuming it after a few seconds which led to a lower final temperature and to a higher quality RNA.

Furthermore, increasing the time of the beating process resulted in a lowering of the total RNA quality. In fact, the attempts made to increase the yield by increasing the beating periods produced degradation of the RNA. This was probably due to a slight increase in temperature and the buffer being unable to completely inhibit the RNase

activity. Thus, the RNA was degraded by endogenous RNases released from the fungal biomass. The second hurdle is how to separate the nucleic acid of interest from the highly complex mixture of proteins, polysaccharides and other mycelia debris resulting from the extraction procedure and still avoiding RNA degradation. In silica membrane column purification is a widely used purification method in many purification kits. However, contamination with polysaccharide can reduce their efficiency significantly (Gambino *et al.*, 2008). Thus a pre-filter column can be used to retain some contaminants and improve homogenization of the lysate. Thus we employed Qiagen's Qiashredder columns. These columns allowed an initial clean-up of the samples which lead to a higher purity of the final eluate. Initially we used TRIzol® reagent during the lysis step, as it is a recognised RNA stabilizer while still holding lysing properties, facilitating cell disruption and dissolving cell components. TRIzol® reagent is a monophasic solution of phenol and guanidine isothiocyanate first described by Chomczynski and Sacchi (1987) that has been extensively used for the isolation of total RNA from cells and tissues with good results.

TRIzol® reagent was later compared with the lysing buffer provided with the RNeasy Plant mini kit, the RLT buffer. Wang *et al.* (2008) showed an increase in RNA quality and in some applications RNA yield by using TRIzol® reagent in conjunction with the Qiagen RNeasy Mini Plant kit. In our studies, on the other hand, the RLT lysis buffer gave similar quality total RNA with a slight increase in yield, which might be due, to some extent, to the improved lysing effect on the ground fungal biomass during the bead beating process. The RLT buffer is based on guanidine thiocyanate salts. This buffer was supplemented with β-mercaptoethanol which irreversibly denatures RNases. To improve the RNA quality, polyvinylpyrrolidone (PVP) which can inhibit the polyphenol oxidase of nucleic acids that result on their degradation can also be used (Abdel-Hadi *et al.*, 2010; Chan *et al.*, 2007).

This second step could still be further improved by using cationic surfactants like cetyl trimethylammonium bromide (CTAB) which could still provide a slight improvement on the total RNA yield as suggested by Wang and Stegemann (2010). However, they were extracting RNA from human mesenchymal stem cells from rich polysaccharide matrices. Nevertheless, the low yield of DNA achieved with *Fusarium* species would

suggest that it might not improve the total RNA yield from filamentous fungi (Fredlund *et al.*, 2008). This method would also be too laborious to be considered as a high throughput system.

Fredlund *et al.* (2008) reported more consistency in DNA yield when extracting from *Fusarium* species, when using an automatic robotized method. The use of an automated method for purification allows a decrease of sample handling time, less chance of cross contamination risk and reduced variability. This enables a comparison to be made between the different extraction methods and conditions during RNA purification. We used the QIAcube (Qiagen) automated platform in our studies.

This showed that it was possible to develop a fast and reliable method to produce high quality RNA that can be subject to functional genomic studies by RT-qPCR. We followed the MIQE guidelines (Bustin *et al.*, 2009) while developing this method. This is important for generating significant and relevant data from RT-qPCR experiments. Indeed, the first step to generate good reliable data is to be able to extract high quality nucleic acids. In developing a systems approach linking molecular, ecology and physiology data, a high throughput system is essential to generate the necessary data. With the method developed in this study it will be possible to process a large number of samples in a shorter period of time, improving total RNA yields and overall quality comparing to the traditional method it requires nevertheless the use of specialized bead beaters. The use of the QIAcube could be substituted by manual purification methods reducing the cost and increasing optimization parameters even though sample handling, variability and risk of cross contamination would also increase.

Even though our reported protocol focuses on high quality RNA, it could potentially be adapted to DNA extraction and help in the large scale genomic studies in filamentous fungi to take fully advantage of their biotechnology capabilities (Meyer, 2008). This method has potential for use in microarray studies where quality and integrity of RNA is critical to obtaining relevant results.

4 The impact of anti-fungal compounds and environmental stress on *Fusarium graminearum* and trichothecene production and expression of *Tri5* biosynthetic gene using RT-qPCR.

4.1 Introduction

Fusarium graminearum is a plant pathogen responsible for Fusarium head blight disease (FHB) which causes significant worldwide losses of cereals, especially wheat. Additionally *F. graminearum* is a trichothecenes type B producer, especially deoxynivalenol (DON). Primarily *F. graminearum* infection of the plant is concurrent with the anthesis period and thus predominantly produced prior to harvest (Cleveland *et al.*, 2003; Miller, 2008). Nonetheless, contamination may also occur during harvesting or post-harvest if poor drying regimes are used or the storage conditions are not adequate (Wagacha and Muthomi, 2008).

In Europe, DON is the predominant trichothecene contaminant and EU legislation limits exist. The Joint FAO/WHO Expert Committee on Food Additives (JECFA) estimates the human dietary intake of DON in Europe to be 1.4 µg/kg bodyweight per day which exceeds the recommended tolerable daily intake (TDI) of 1 µg/kg bodyweight per day. Interestingly in Africa the dietary intake of DON is estimated to be lower than the recommended TDI because of the predominance of maize in the diet (Meneely *et al.*, 2011; Turner *et al.*, 2008; Pestka, 2010).

Trichothecenes, like many other mycotoxins, are very stable molecules and detoxification is not easily achieved. Trichothecenes require temperatures in the region of 210ºC to decompose and chemical treatments to deliberately detoxify foodstuff is not allowed by the EU (European Commission, 2006; JECFA, 2001). Physical methods of removing contaminated grain during harvesting and during post-harvest storage or during milling are measures that can be adopted to reduce mycotoxin contamination in the food chain. Nevertheless, the accumulation of trichothecenes must be controlled through the entire food chain. In order, to control trichothecenes accumulation the conditions that allow *F. graminearum* growth must be avoided and

more importantly factors that contribute to the upregulation and expression of the biosynthetic trichothecene genes need to be controlled. Recent studies by Schmidt-Heydt *et al.* (2011) showed the effect of changes in water availability x temperature influenced the expression of key genes in the trichothecene biosynthetic pathway.

The trichothecene biosynthetic genes in *F. graminearum* are clustered together. The 25 kb cluster is composed of 12 co-regulated genes with 10 of them being required for trichothecene biosynthesis located on chromosome 2. A further 3 additional genes, *Tri1*, *Tri101*, and *Tri15*, are located on chromosomes 1, 4, and 3, respectively (Hallen-Adams *et al.*, 2011). *Tri5* gene encodes for trichodiene synthase which catalyses isomerization of farnesyl pyrophosphate to form trichodiene, a key step of the trichothecene biosynthetic pathway and has been correlated to DON phenotypic expression (Doohan *et al.*, 1999; Schmidt-Heydt *et al.,* 2011). Some recent studies have reported that *Tri5* expression in *F. graminearum* appears to be constant within a wide range of water activity and temperature range (Marín *et al.*, 2010) and that *Tri5* expression in wheat had an expression peak concurrent with the infection front (Hallen-Adams *et al.*, 2011). *Tri5* expression peak at the infection front is concordant with a role that trichothecene may have in the ability of *F. graminearum* to infect wheat and cause FHB (Alexander *et al.*, 2009).

Environmental factors have a profound effect on production of mycotoxins such as DON. The most important factors controlling fungal growth in foodstuffs are temperature, water activity (a_W), pH or intergranular gas composition depending on the food matrix in question (Magan, 2007; Magan and Aldred, 2007; Magan *et al.*, 2010). There is interest in examining the impact that interactions between anti-fungals (butylated hydroxyanisole, prochloraz and thyme essential oil) and water availability have on growth and *Tri5* gene expression and phenotypic mycotoxin production. Studies by Schmidt-Heydt *et al.* (2009) showed that intermediate concentrations of propionate preservatives can inhibit growth but stimulate ochratoxin A production by *P. verrucosum* and this was confirmed by examining the expression of the *otapks*Pv gene which was correlated with ochratoxin A production. No such knowledge exists for *F. graminearum* and intermediate concentrations of potential control compounds.

The aims of this study were (a) effect of intermediate concentrations of an essential oil, fungicides and anti-oxidant on growth of *F. graminearum* under different a_W x temperature conditions, (b) evaluate the impact of environmental factors and the presence or absence of anti-fungal compounds have on the expression of *Tri5* gene and c) the effect that these compounds at intermediate concentrations have on type B trichothecene production (DON, Nivalenol, 15 and 3 AcDON) and their ratios.

4.2 Material and methods

4.2.1 *F. graminearum*

F. graminearum isolate I_3 L1-2/2D was isolated from wheat and is a known high producer of DON (Marín *et al.*, 2010). *F. graminearum* isolates WC/08/031, WC/08/091, WC/08/111 and WC/08/133 were also examined and kindly supplied by Professor Simon Edwards, Harper Adams University College, Newport, UK. The cultures were maintained on Malt Extract Agar (MEA) media (OXOID, malt extract, 30; mycological peptone, 5; agar, 15 g/L).

4.2.2 Trichothecenes type b expression artificial media and grain

Trichothecene expression was induced *in vitro* by sub-culturing all cultures of *F. graminearum* on Yeast Extract Sucrose (YES) medium (20.0 g/L Yeast extract, 150.0 g/L Sucrose, 15 g/L Agar) (Davis, *et al.* 1966).

For the *in vitro* studies, 20 ml of YES medium was poured into 9 cm Petri plates. After cooling, a sterile 90 mm disc of cellophane was placed aseptically onto the agar surface avoiding puncturing the cellophane surface. This facilitated later removal of the mycelia colony for molecular analyses.

Gamma irradiated wheat was used to examine *in situ* effects of anti-fungal compounds on growth, *Tri5* and trichothecene production. The grains were irradiated at 12 kGy (Isotron, Plc, Swindon, UK) in order to retain germinative capacity but be free of fungal contamination (Hope et al., 2005) and they were stored at 4°C until further use.

A moisture adsorption curve was used to determine the accurate amounts of water required to obtain the target a_W levels for the experiments. These are detailed below. Approximately 5 g of water activity modified irradiated grain was dispersed on 50 mm Petri dishes to obtain a monolayer on which a 4 mm agar plug from the edge of a growing culture could be inoculated. These were stored in plastic chambers containing glycerol/water solutions to maintain the ERH at the equivalent a_W level of the irradiated grain.

4.2.3 Water activity adjustments

The water activity of the YES agar media was adjusted using 1.50, 9.65 and 18.50g of glycerol per 100ml of media, to attain respectively, 0.98, 0.96 and 0.94 a_W. The final a_W of the media was measured using an Aqualab® 3TE (Aqualab, USA) water activity analyser. Glycerol/water solutions were used to calibrate the accuracy of the water activity levels (Table 4.1).

Table 4.1- The relationship between molality and a_W of aqueous glycerol solutions and to the amount of glycerol (g) per 100 ml of water for the preparation of aqueous glycerol solutions.

Molality	a_W	Amount of glycerol (g) added to 100 ml of RO water
1.0	0.982	9.209
2.0	0.964	18.419
3.5	0.937	32.233

Treatments of the same a_W were kept in separate plastic bags to maintain the conditions during the experimental period.

Known amounts of sterile water were added to the irradiated grain according to a water adsorption curves previously developed and allowed to equilibrate for 72 h. A roller shaker was used to allow uniform adsorption to a final a_W of 0.98, 0.96 and 0.94 for the irradiated grain treatments. The equilibrated wheat final a_W was measured using the

same method as for the YES medium. All measures were within 0.005 a_W of the target values.

4.2.4 Inoculation and incubation

Cultures of the *F. graminearum* strains were prepared on MEA and incubated at 25°C for 10 days. Spores were collected using a sterile loop and placed in a sterile 25 mL Universal bottle containing 10 mL of reverse osmosis (RO) water (Direct-Q 3 UV, Millipore, France) with 0.05% Tween 80 (ACROS organics). The spore suspensions was counted using a haemocytometer (Olympus BX40 microscope, Microoptical Co.; slide Marienfeld superior, Germany; microscope glass cover slips, No 3, 18×18mm, Chance proper LTD, UK) and adjusted to $1x10^6$ spores per mL.

Petri plates with glycerol modified YES were inoculated with 10 µl of the spore suspension and incubated at 20, 25 and 30ºC. The experiments were carried out with three replicates per treatment.

4.2.5 Growth assessment

For each replicate the radial growth was measured in two directions at right angles to each other with a ruler. The growth rate was calculated based on the slope of the linear region of the growth curve for each replicate.

4.2.6 Preparation of stock solutions of the antifungal compounds

Stock solutions of butylated hydroxyanisole (BHA), prochloraz and thyme essential oil were prepared in sterile distilled water. The solutions were then filter-sterilised through a sterile 0.2 µm Millipore filter (Minisart, Sartorius) into sterile containers. When higher concentrations of the compounds were required or when the solubility of the compounds did not allow the preparation of water solutions, stock solutions were prepared in 50% absolute ethanol (HPLC Grade). These were incorporated into the agar media or added to the wheat irradiated grain to take account of the target a_W levels for the treatments. Suboptimal concentrations of antifungal compounds can

lead to an increase in mycotoxin production in other mycotoxigenic organism. A summary of the suboptimal conditions used is shown at Table 4.2.

Table 4.2 - Treatment conditions used in this study

Compound	Final concentration		Glycerol modified a_W	Temperature
	In vitro	*In situ*		
Control	-	-	0.98 0.96 0.94	25ºC
Control + Ethanol	0.3 ppm	1.6 ppm		
BHA	50 ppm + 0.3 ppm Ethanol	250 ppm + 1.6 ppm Ethanol		
Prochloraz	0.1 ppm	5 ppm		
Oil of Thyme	0.2 ppm	200 ppm		

4.2.7 Trichothecene analyses

The wheat irradiated grain samples were oven-dried at 60°C for 24-48 h, milled in a small laboratory blender and analysed for trichothecenes type B by Liquid Chromatography Tandem Mass Spectrometry (LC-TMS) by the department of Agrobiotechnology of the University of Natural Resources and Applied Life Sciences, IFA-BOCU, Tulln, Vienna, Austria. Half of the milled *in vitro* samples were used to develop an HPLC method to analyse trichothecenes type B.

4.2.8 Molecular analyses

The fungal biomass was quickly frozen in liquid nitrogen and stored at -80ºC until RNA extraction could be carried out.

To increase efficiency and decrease the variability on the RNA yields, an automatic total RNA extraction and purification system was used (see Chapter 3).

4.2.9 Reverse transcriptase PCR

The amount of total RNA isolated from the fungal mycelia was equilibrated so that a total of 1 µg RNA was reverse transcripted into cDNA using the Omniscript® Reverse Transcription kit (Qiagen, Germany). The total RNA mixture also includes ribosomal RNA, to ensure that only mRNA was transcribed to cDNA, Oligo-dT primers were used. These primers anneal selectively to the polyA tails, characteristic of eukaryotic mRNA. The resulting cDNA was used directly for the Real time qPCR, or stored at -20ºC until use.

4.2.10 Gene expression

The formation of trichodiene is a key biosynthetic step in the production of trichothecenes. *Trichodiene synthase (Tri5)* gene has been previously associated with the production of trichothecenes for this reason the gene expression of *Tri5* was accessed by RT-qPCR using the set of primers in Table 4.3.

Table 4.3 - *F. graminearum trichodiene synthase (Tri5)* gene primers, gene accession number AY130290.1.

Name	Sequence	Position
PQTri5-MF	5' – GAT CTG ATG ACT ACC CTC AAT TCC TT	577
PQTri5-MR	5' – GCC ATA GAG AAG CCC CAA CAC	648

This primer pair produces a 71bp fragment

A different set of amplicons were also amplified, targeting one reference gene in order to normalise the expression levels between the different experiments and samples. The pair of primers in Table 4.4 were used no normalise the gene expression.

Table 4.4 - *F. graminearum β-tubulin* gene primers, gene accession number AY303689.1

Name	Sequence	Position
TUB2PQ-PF	5' – CCG AGG CCC AGT CCA ACT	1513
TUB2PQ-PR	5' – GGC GTC TTG GTA TTG CTG GTA	1571

This primer pair produces a 59bp fragment with *F. graminearum*

For the generation of the standard curves a pool of total RNA from different samples was reversibly transcripted into cDNA. The cDNA pool was 10 fold diluted starting from the equivalent transcripted amount of 2 µg total RNA quantified by spectrometry.

The analysis of the standard curves shows the gene efficiency correction used with the Pfaffl method of gene quantification (Figure 4.1). This method takes into account differences of amplification efficiencies from each reaction to normalize Cycle Threshold (CT) differences in relative quantification. Relative quantification allows the normalization of gene expression with differences of mRNA material input. The initial differences of mRNA amounts can be normalized due to a stable expressed gene. *β-tubulin* was used as a reference gene its expression is assumed to be stable at the wide range of conditions tested and so differences in the quantification of this amplicon relates to initial differences of mRNA quantities and the levels of *Tri5* were normalized against them.

Furthermore the analysis of the melt curve allowed the assessment of the level of specific and overall quality of the PCR reaction, since each peak should represent one amplicon and the presence of multiple peaks might suggest non-specific binding of the primers or even primer-dimer structure and amplification deficiencies (Figure 4.2). After the analysis of the melt curve, the amounts of *Tri5* gene were quantified.

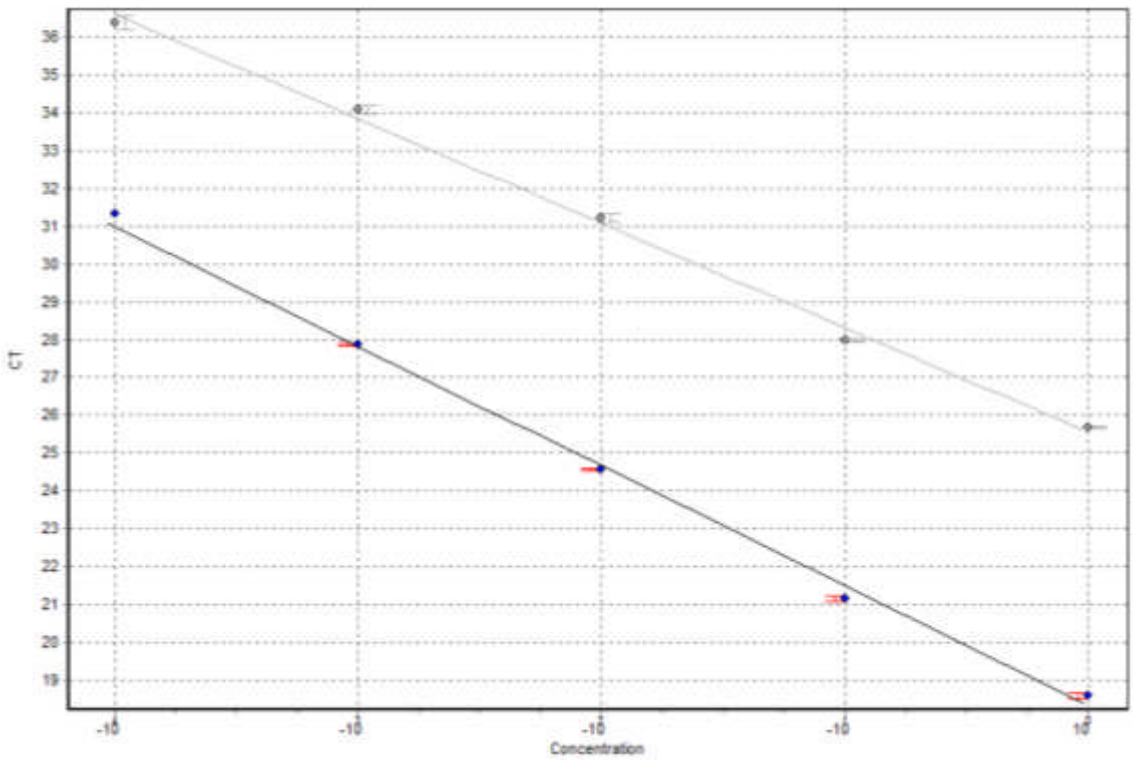

Figure 4.1 - Standard curves used to calculate amplification efficiency for each gene (*Tri5* and *β-tubulin* as reference gene) for relative quantification using the Pfaffl method. Light grey line represents *Tri5* standard curve while darker line represents *β-tubulin* standard curve. The vertical lines represent the mean standard error.

Bioinformatic Q-gene application was used to calculate normalized gene expressions the CT values for both *β-tubulin* and *Tri5* gene were obtained using the Rotorgene Q (Qiagen) software. These values were transferred to the Q-gene application, were Equation 4.1 was used to calculate normalize expression (NE).

$$NE = \frac{(E_{\beta\text{-tubulin}})^{CT_{\beta\text{-tubulin}}}}{(E_{Tri5})^{CT_{Tri5}}}$$

Equation 4.1 - Equation used by Bioinformatic Q-gene application to calculate normalized expression taking into account primer amplification efficiency (E), equation adapted from Simon (2003)

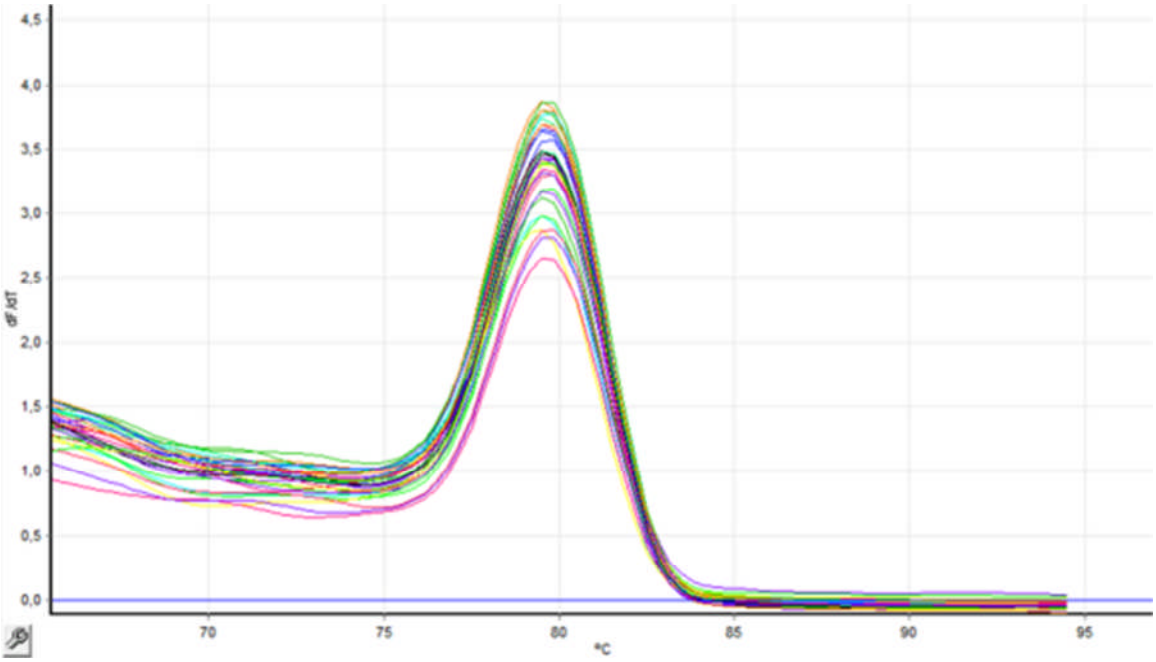

Figure 4.2 - Melting curve analysis of *Tri5* amplicons obtained after the RT-qPCR reaction.

4.3 Results

4.3.1 Effect of environmental factors and anti-fungals on growth of *F. graminearum* strains.

(a) In vitro

Initial growth rate was assessed for all strains to determine possible strain differences (Figure 4.3). This showed that there was no significant difference between the strains examined and we used one strain as a representative example for the rest of the studies.

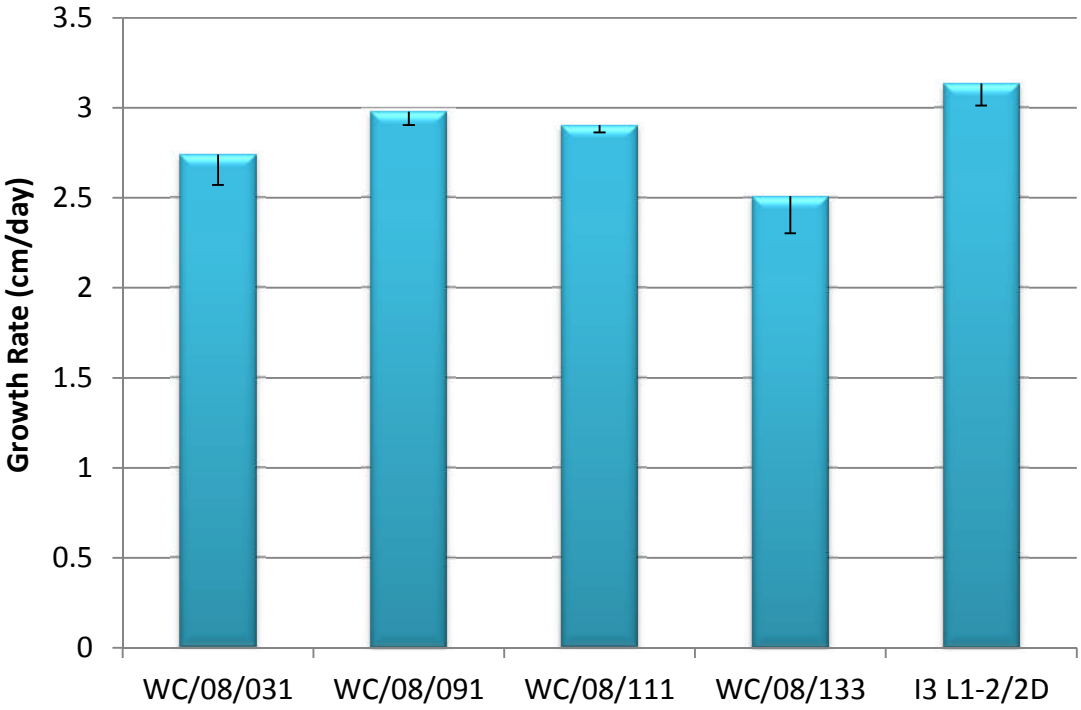

Figure 4.3 - Comparison of the growth rate of different strains of *F. graminearum* at 25ºC on YES. Vertical lines indicate standard error.

The effect of a_W x temperature conditions on growth of the strain of *F. graminearum* used in subsequent studies is shown in Figure 4.4. This shows that the fastest growth was at >0.98 a_W and 25ºC.

Figure 4.5 shows the effect of the anti-fungal compounds (BHA, Prochloraz and thyme oil) at sub-optimal concentrations on the growth of *F. graminearum*. This shows that the effect of these compounds was variable and was affected by a_W treatments.

Table 4.4 summarises the relative level of inhibition obtained with the different antifungal compounds in relation to the different a_W levels examined.

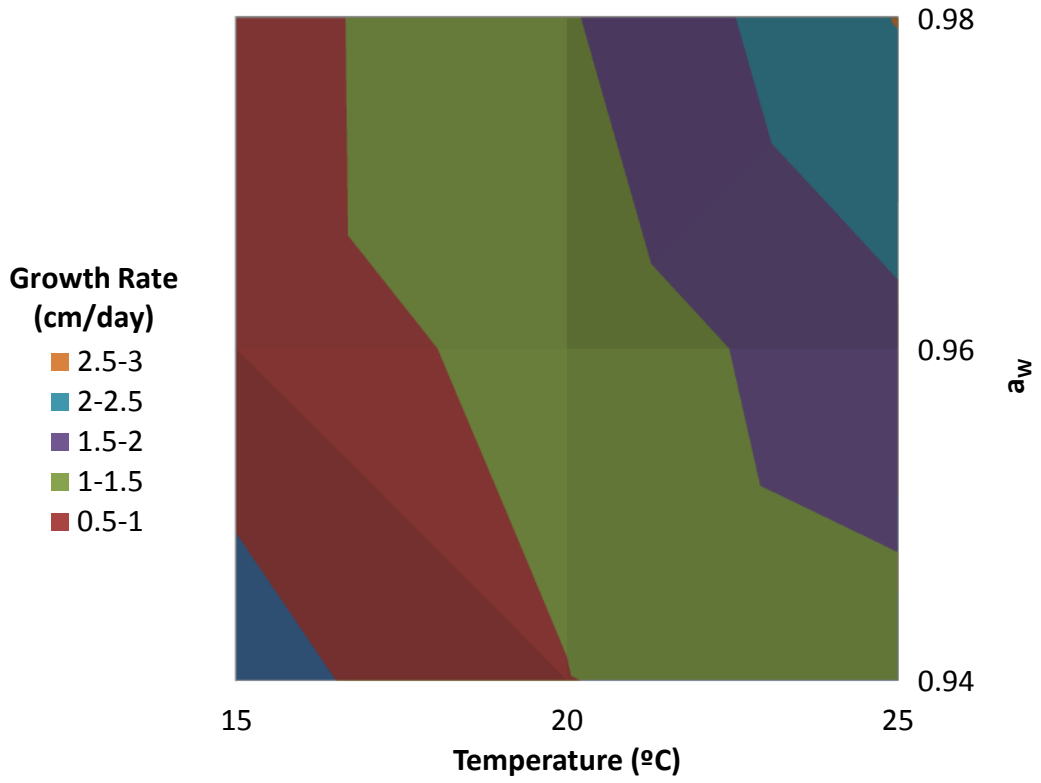

Figure 4.4 - Contour map of the effect of water activity x temperature effects on the growth of *F. graminearum on a YES medium.* Colours indicate the regions for different growth rates.

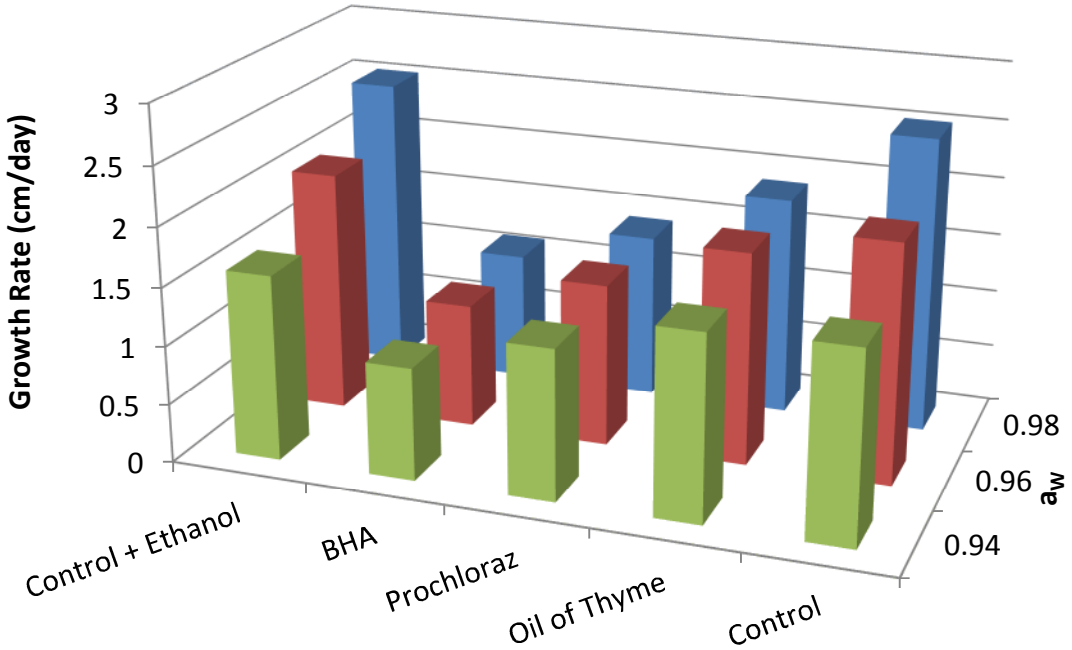

Figure 4.5 - Effect of anti-fungal compounds on growth of *F. graminearum* under different water stress conditions at 25ºC.

Table 4.5 - Inhibition of the growth rate at 25ºC by different antifungal compounds and water activity. Reduction of the growth rate of the compounds is related to the growth rate at the same water activity of the control.

Condition / a_w	a_w	BHA	Prochloraz	Oil of Thyme
0.98		56%	44%	26%
0.96	19%	49%	32%	11%
0.94	36%	39%	20%	2%

(b) In situ

The impact of anti-fungal compounds on growth of *F. graminearum* under different water stress conditions at 25ºC were assessed *in situ* on wheat irradiated grain (Figure 4.6). This shows that the efficacy *in situ* was not as effective as *in vitro* and this varied with antifungal compound used. At 0.94 a_w treatments completely inhibited growth of the *F. graminearum* strain used.

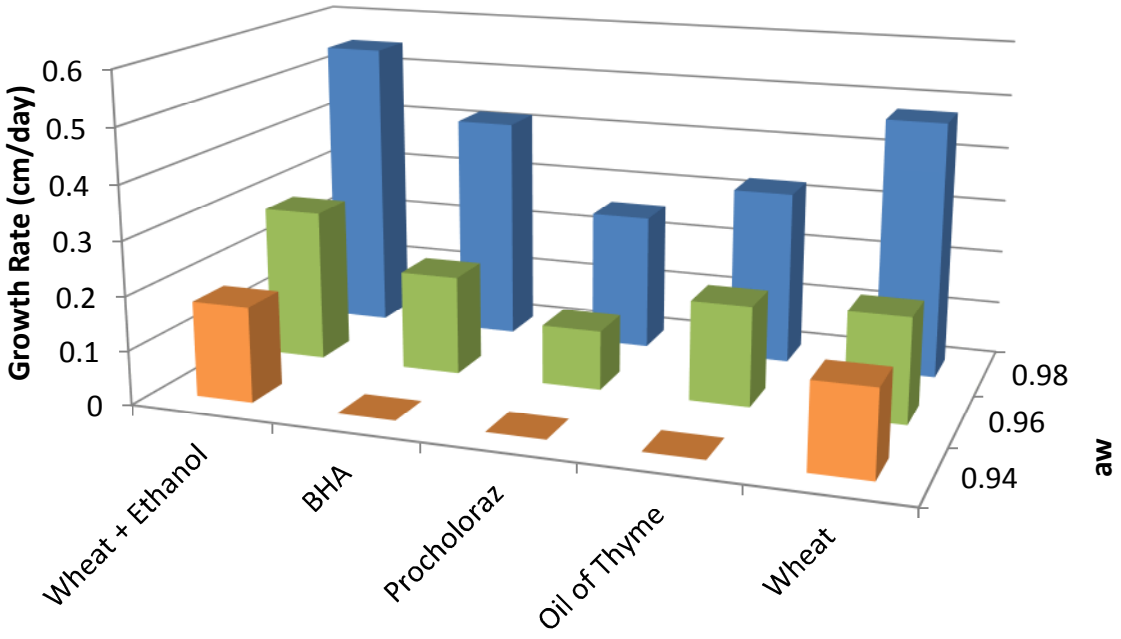

Figure 4.6 - Influence of anti-fungal compounds on growth of *F. graminearum* in wheat under different water stress conditions at 25ºC.

Plate 4.1 shows the influence of water activity over 3, 6 and 9 days on *F. graminearum* cultures grown on moisture irradiated wheat.

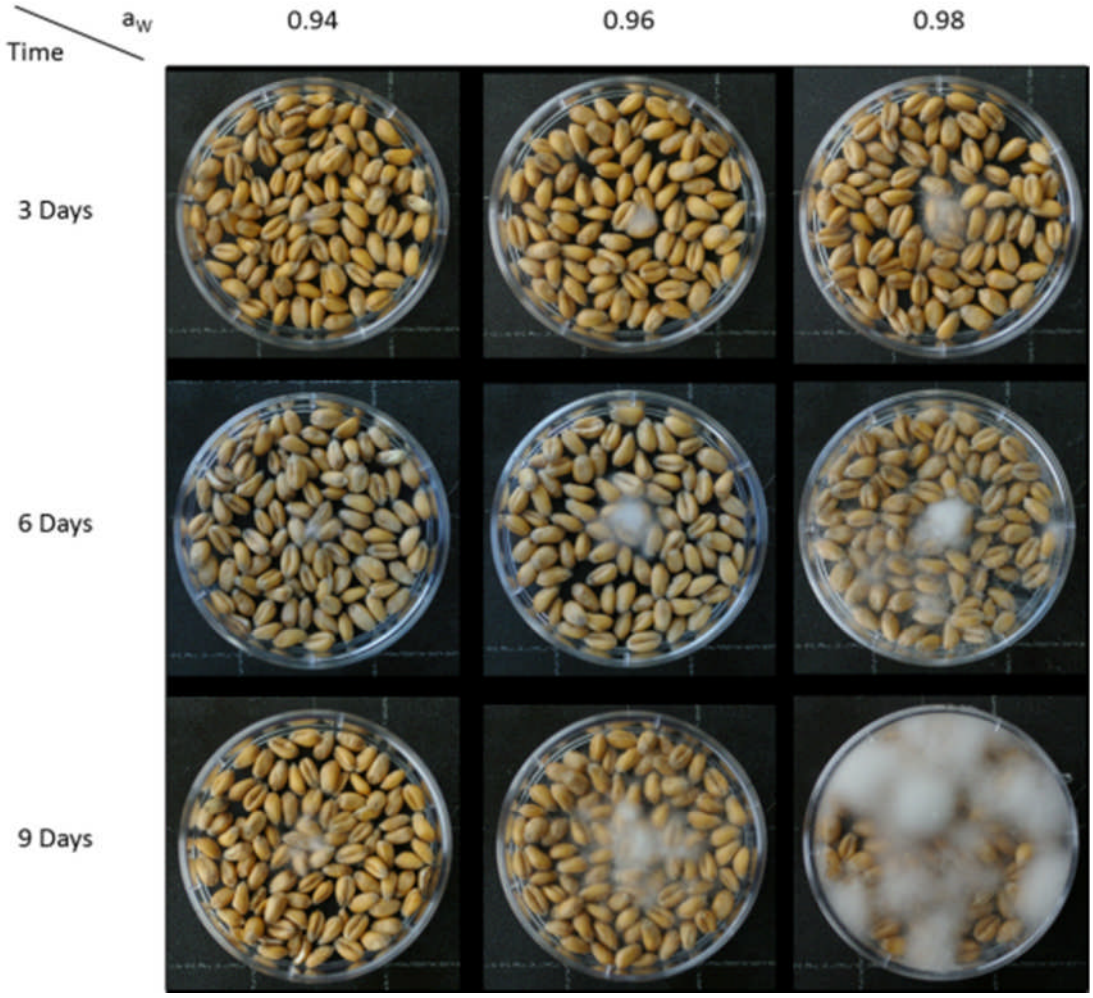

Plate 4.1 - Influence of water activity on the growth of *F. graminearum* over 9 days.

Plate 4.2 shows the influence of the interaction of ethanol at the same conditions used in the stock solutions of BHA and water activity over 3, 6 and 9 days on *F. graminearum* grown on irradiated wheat, This plate serves as control for the BHA effect.

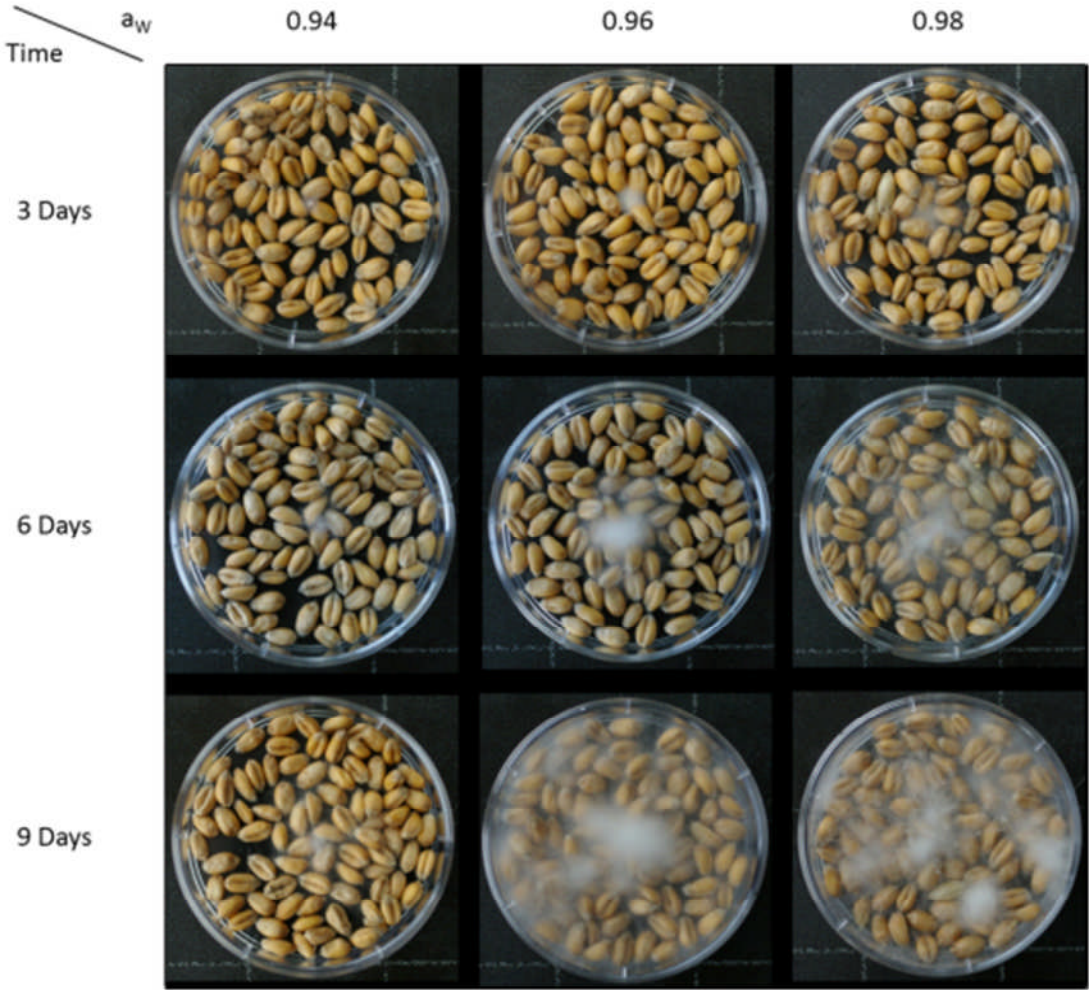

Plate 4.2 - Interactions of ethanol and different water activity levels on the growth of *F. graminearum* over 9 days.

Plate 4.3 shows the influence of the interaction of BHA and water activity over 3, 6 and 9 days on *F. graminearum* cultures grown on moisture irradiated wheat.

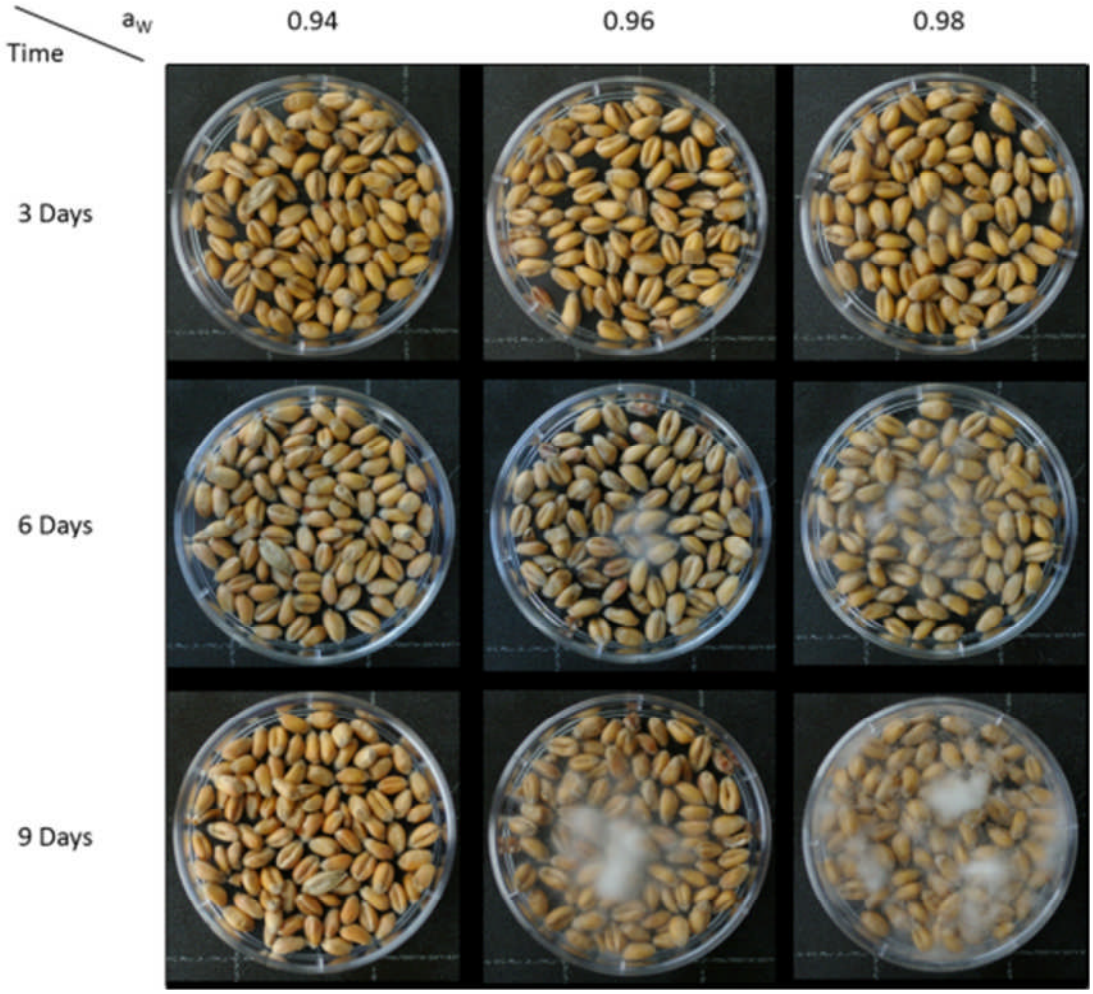

Plate 4.3 - Interactions of BHA and different water activity on the growth of *F. graminearum* over 9 days.

Plate 4.4 shows the influence of the interaction of prochloraz and water activity over 3, 6 and 9 days on *F. graminearum* cultures grown on moisture irradiated wheat.

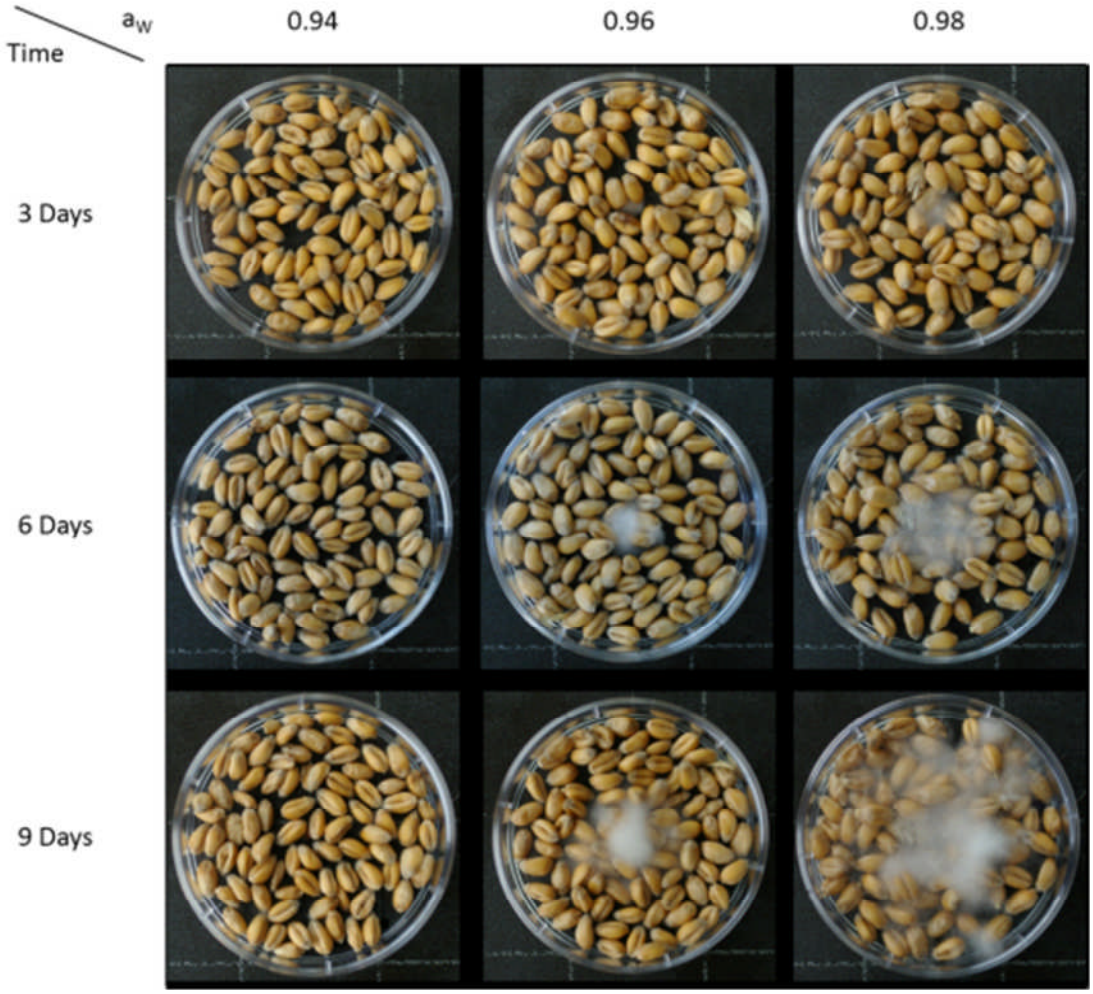

Plate 4.4 - Interactions of prochloraz and different water activity on the growth of *F. graminearum* over 9 days.

Plate 4.5 shows the influence of the interaction of thyme essential oil and water activity over 3, 6 and 9 days on *F. graminearum* cultures grown on moisture irradiated wheat.

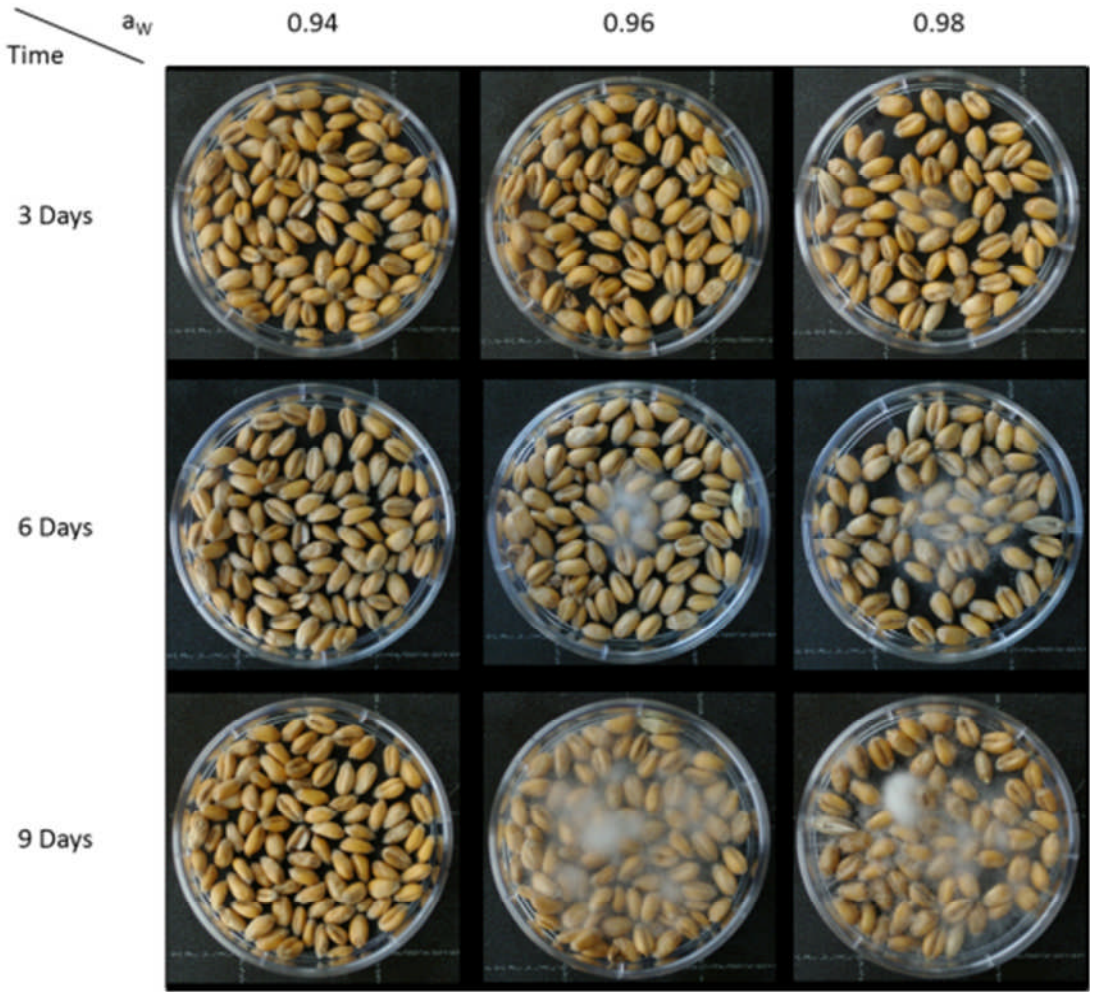

Plate 4.5 - Interactions of thyme essential oil and different water activity on the growth of *F. graminearum* over 9 days.

Equilibrated irradiated grain to the different water activity tested was incubated at 25ºC for several weeks to assess possible remaining natural mycoflora. All irradiated grain was sterile. No growth was observed in the irradiated wheat or when the irradiated grain was cultured in MEA media.

4.3.2 *F. graminearum Tri5* gene expression

(a) Comparison of *F. graminearum* strains for *Tri5* gene expression.

Figure 4.7 compares the relative expression of the *Tri5* gene by the 5 different strains. This shows that there was some variation between the expressions found in the strains. This shows that most of the strains there was no significant differences

between them. The strain of *F. graminearum* I_3 L1-2/2D had the higher level of *Tri5* gene expression and the subsequent studies were carried out using this strain.

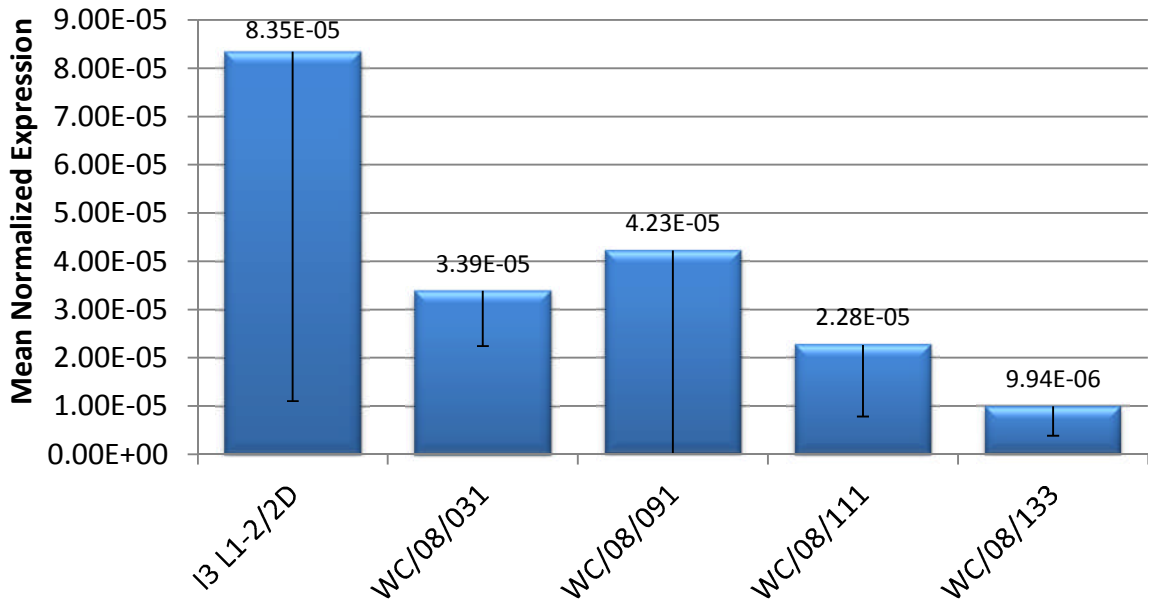

Figure 4.7 - Strain differences of normalized *tri5* gene expression levels. All strains were cultured on YES agar media at 25ºC for 9 days. Vertical lines indicate standard error of the mean.

(b) Effect of anti-fungal compounds on *Tri5* gene expression by the strain of *F. graminearum*

Figure 4.8 compares the effect of BHA on normalized expression of *Tri5* under different a_W levels *in vitro* and *in situ*. This shows that there were differences in expression between a_W levels. However, *in situ* the higher expression in both the control and the BHA treatment was at intermediate water activity levels. This is different form the *in vitro* studies, where the highest expression levels were at different a_W.

Figure 4.9 compares the effect of all the treatments *in vitro* and *in situ*. This shows that *in vitro* there was significant control of *Tri5* gene by the antifungal compounds. However, at the same concentrations there was enhanced *Tri5* expression *in situ* in irradiated grain especially for both thyme oil and prochloraz at 0.98 and 0.96 a_W treatments.

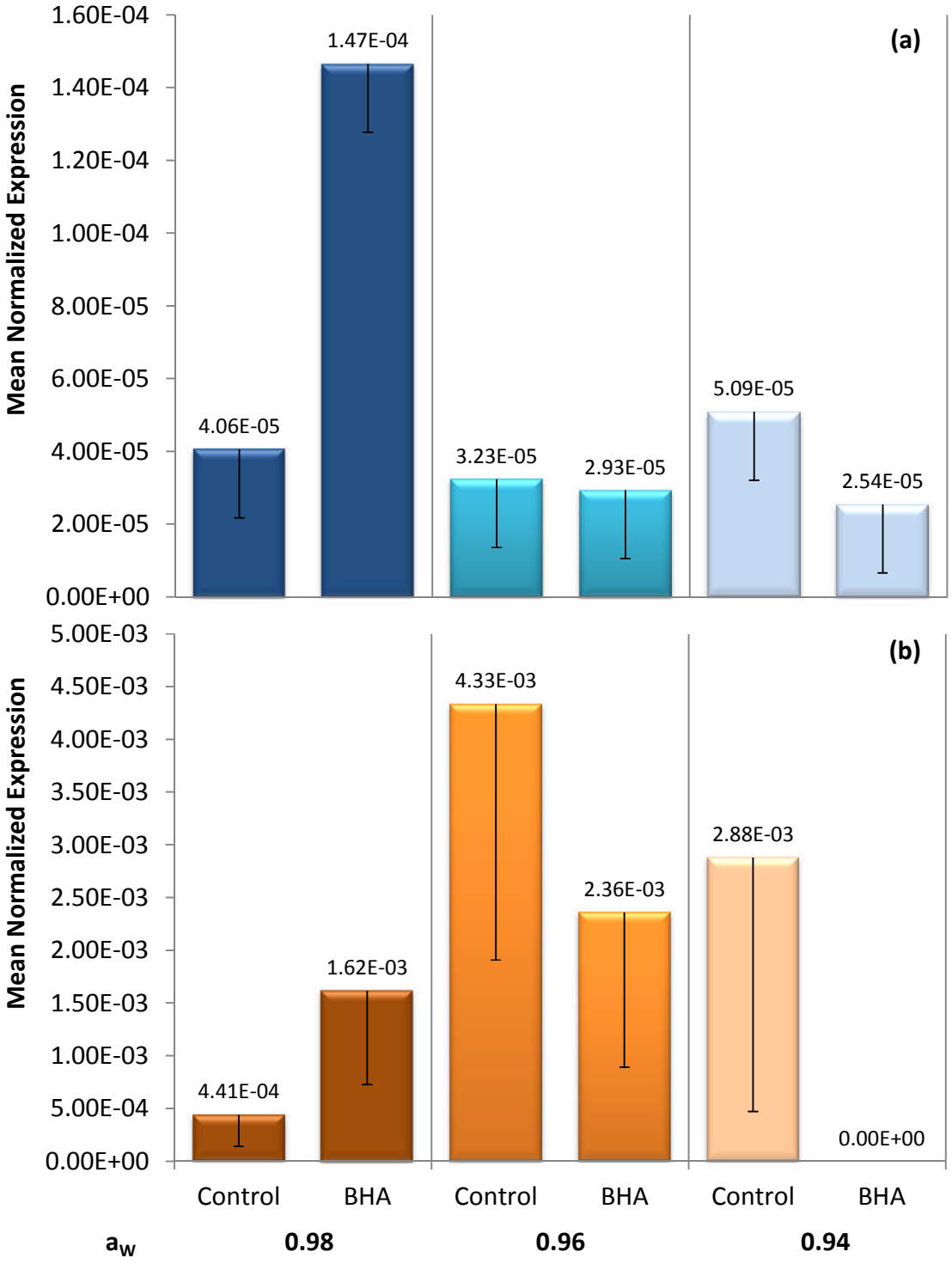

Figure 4.8 - Influence of water stress conditions and BHA on *Tri5* gene expression at different a_W levels. **a)** Blue bars on top represent "*in vitro*" results of normalized *Tri5* expression **(b)** orange bars below represent "*in situ*" results of normalized *Tri5* expression. Darker colours represent 0.98 water activity while lighter colours represent 0.94 water activity. The middle bars represent 0.96 water activity conditions. Vertical lines indicate standard error of the mean.

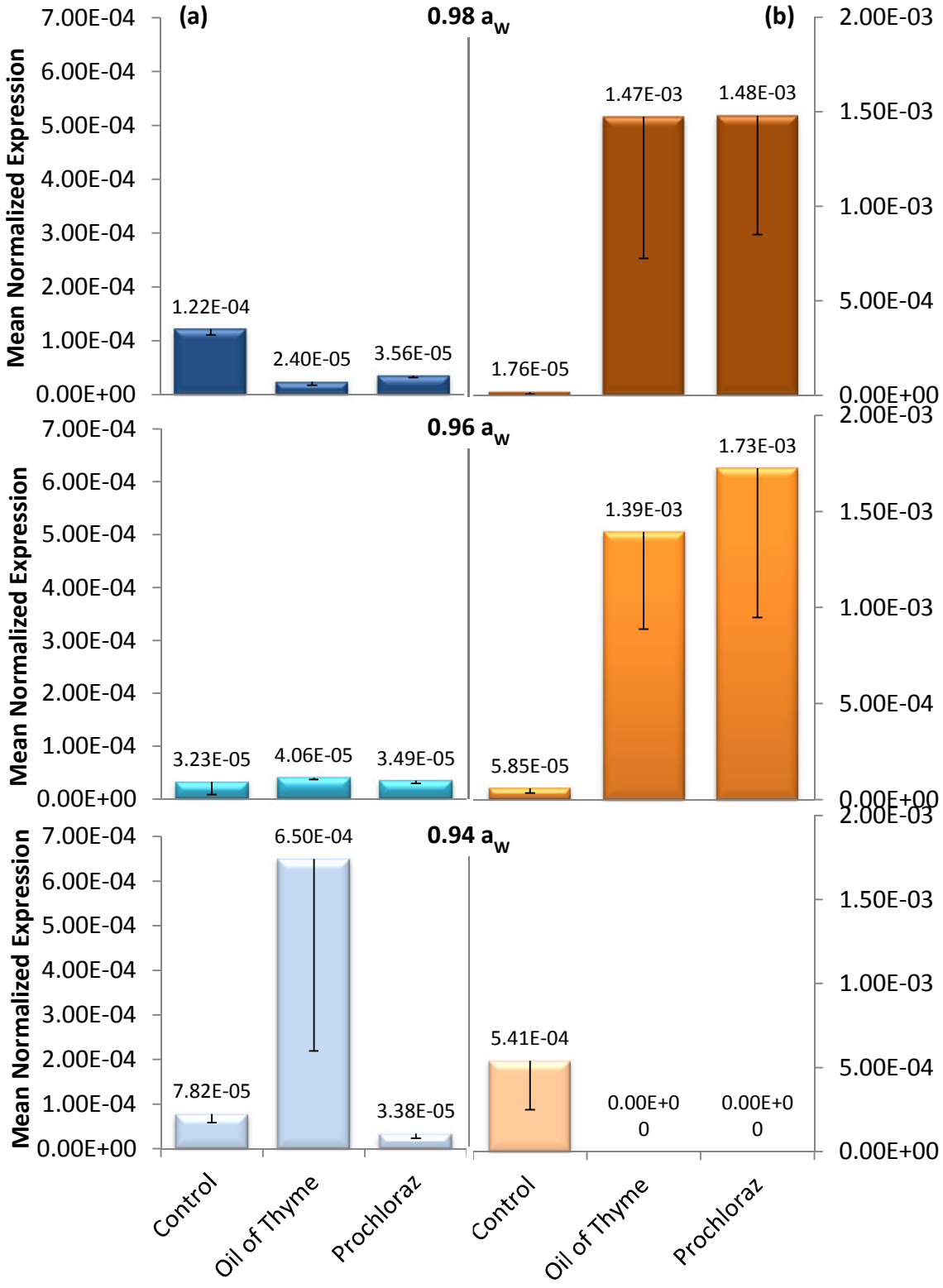

Figure 4.9 - *Tri5* gene expression in relation to antifungal compounds at three different a_W levels. **(a)** Blue bars on the left represent "*in vitro*" results of normalized *Tri5* expression **(b)** orange bars on the right represent "*in situ*" results of normalized *Tri5* expression. Darker colours represent 0.98 water activity while lighter colours represent 0.94 water activity. The middle bars represent 0.96 water activity conditions. Vertical lines indicate standard error of the mean.

4.3.3 *F. graminearum* trichothecenes type B production

Milled wheat irradiated grain contaminated with *F. graminearum* was analysed for the presence of DON contamination after 10 and 20 days, figure 4.10 and 4.11 respectively.

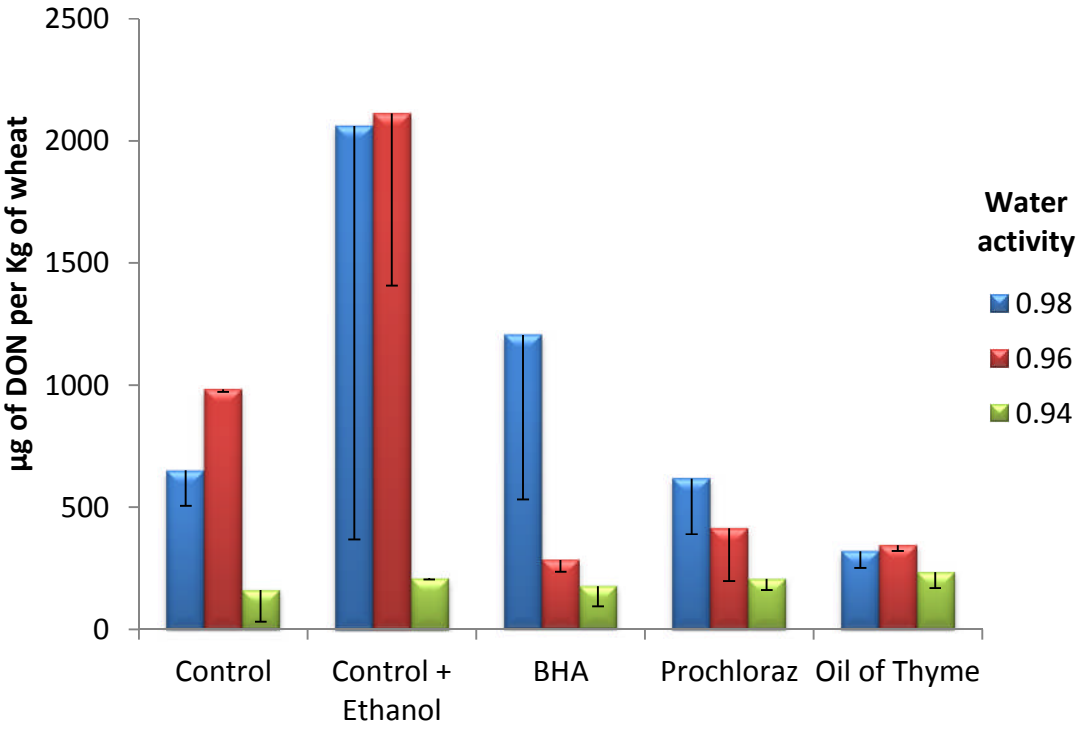

Figure 4.10 - µg of DON per kg of wheat quantified from 10 day wheat samples at different water activities and added compounds. Vertical bars indicate the standard error of the means.

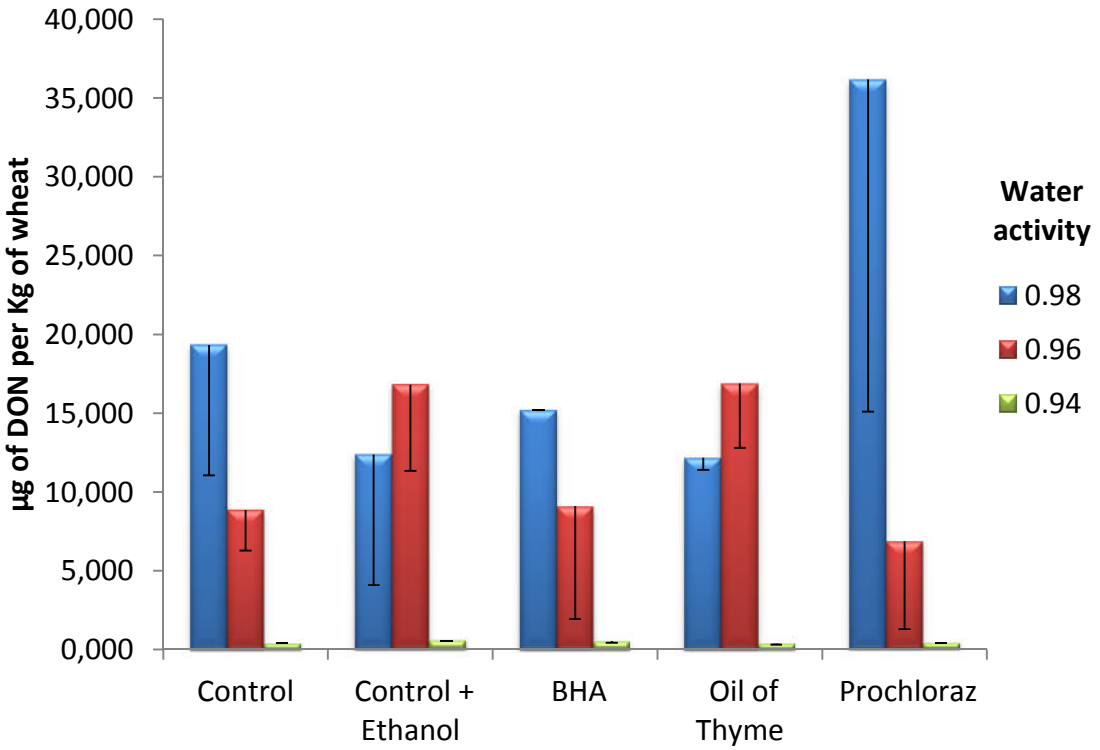

Figure 4.11 - µg of DON per kg of wheat quantified from 20 day wheat samples at different water activities and added compounds. Vertical bars indicate the standard error of the means.

Furthermore, Nivalenol as well as the acetylated forms of 15 and 3 AcDON, also trichothecenes type b toxins were detected at all samples under all conditions and treatments. The cumulative amount of trichothecenes type is shown on Figure 4.12 and 4.13 for the 10 day samples. The 20 day samples are represented on Figure 4.14 and 4.15

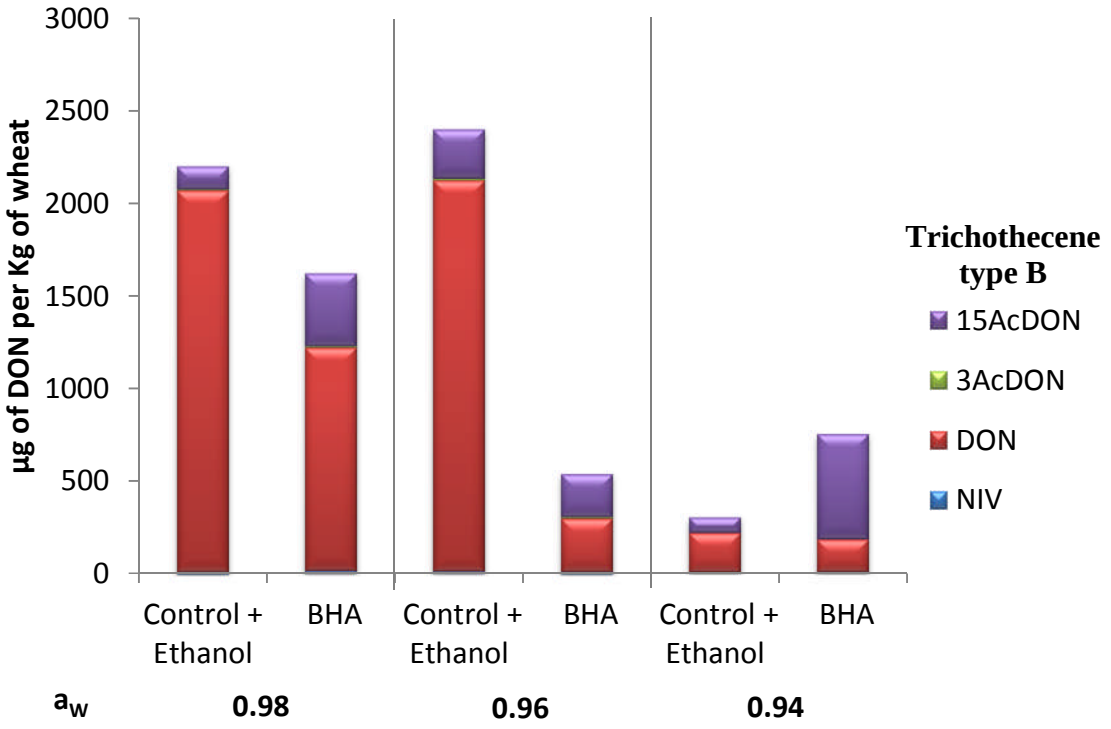

Figure 4.12 - Cumulative µg of trichothecenes type B toxins per kg of wheat quantified from 10 day wheat samples at different water activities and with the presence or absence of BHA.

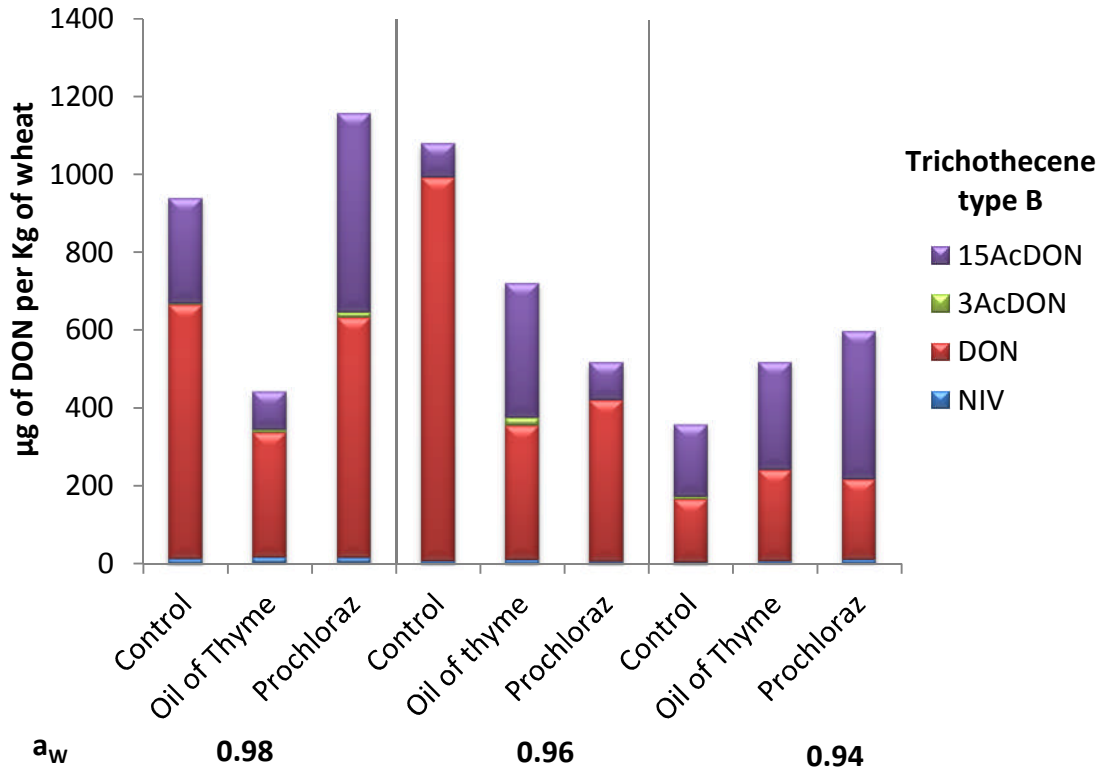

Figure 4.13 - Cumulative µg of trichothecenes type B toxins per kg of wheat quantified from 10 day wheat samples at different water activities and antifungal compounds.

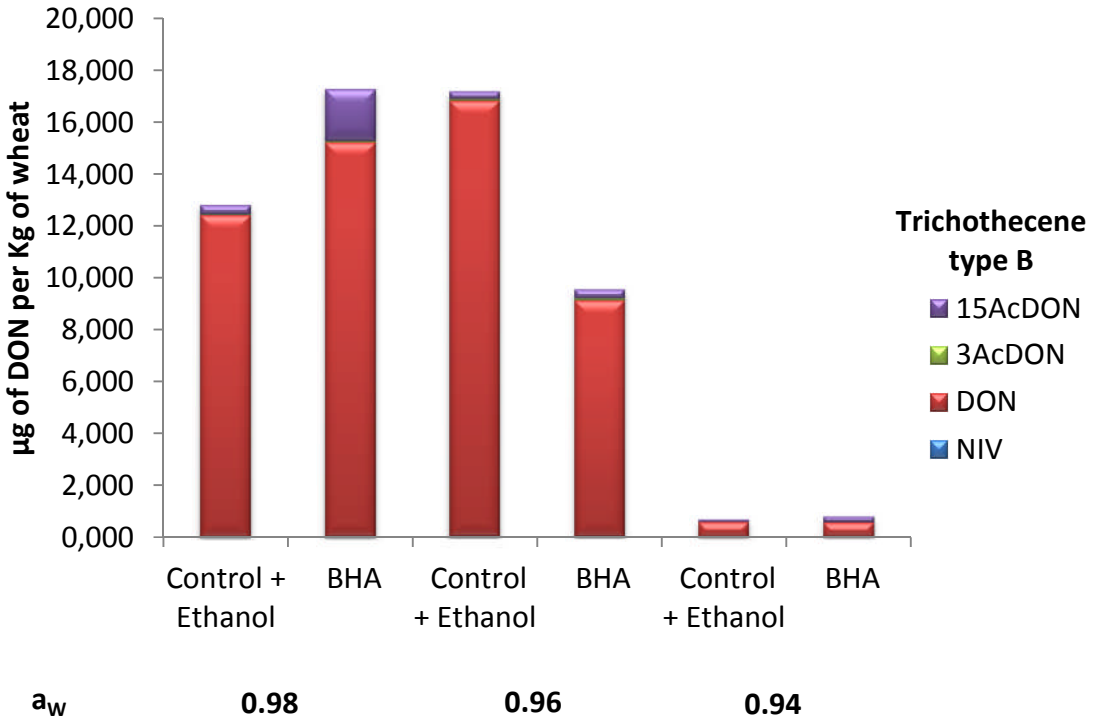

Figure 4.14 - Cumulative µg of trichothecenes type B toxins per kg of wheat quantified from 20 day wheat samples at different water activities and with the presence or absence of BHA.

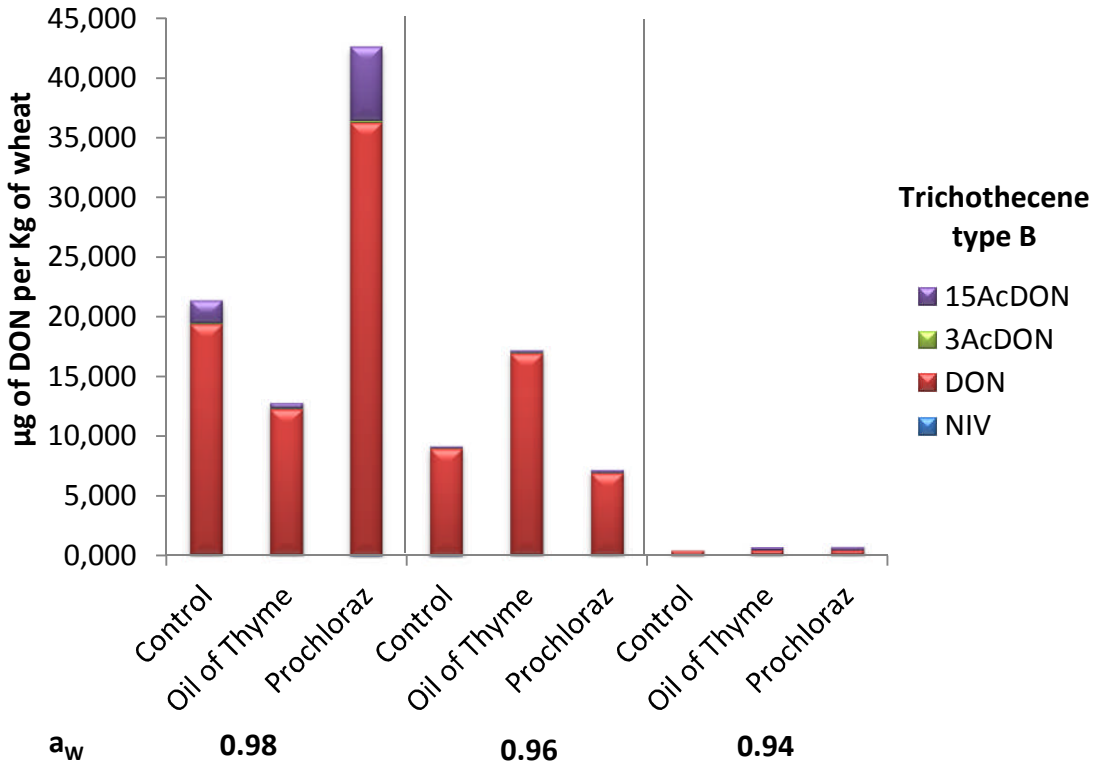

Figure 4.15 - Cumulative µg of trichothecenes type B toxins per kg of wheat quantified from 20 day wheat samples at different water activities and antifungal compounds.

4.4 Discussion

The initial assessment of the growth rate of different strains of *F. graminearum* showed small differences between the studied strains with the exception of the strain I_3 L1-2/2D. All others had a growth rates between 2.50-3.00 cm/day. *F. graminearum* I_3 L1-2/2D showed a growth rate of 3.13 ± 0.12, and this strain was used for the remaining studies.

F. graminearum growth rate was affected by temperature and water activity. Optimum growth was achieved at higher water activities and 25ºC with the growth rate maximum of 2.53 cm per day at 0.98 water activity and a minimum at a water activity of 0.94 of 1.27 cm per day. The temperature reduction led to a reduction in growth rate. At 20ºC the growth rate was between 1.455 and 0.99cm per day this was even lower at 15ºC were the growth rate did not exceeded 0.77 cm per day and when the water activity was lowered to 0.94 growth was further inhibited to 0.29 cm per day.

These results are similar to those reported for the strain I_3 L1-2/2D by Marín *et al.* (2010). The maximum growth rate was reported to be at 25ºC and water activity of 0.98. The growth rate reported in the present study is slightly higher but that is probably due to a richer YES medium compared to the GYEP (glucose, yeast extract, peptone) medium used previously. Furthermore the bi-dimensional growth rate map presented by Marín *et al.* (2010) suggests a maximum growth rate at 25ºC between 0.98 and 0.995 water activity which we also found to be optimum in the present study. The actual minimum a_W levels for growth of *F. graminearum* are closer to 0.88-0.90 aw depending on incubation time at around 25^{o}C (Hope *et al.*, 2005). However, these marginal conditions were not as important in terms of trichothecene production as the a_W x temperature profile for DON production is narrower.

Subsequently the impact of the intermediate concentrations of antifungal compounds was assessed. The results show that at the concentrations used all compounds were able to reduce the growth of *F. graminearum*. Nevertheless, the effect of water activity seemed to reduce by efficacy of the compounds. The growth rate reduction of 19% in the controls from reducing the water activity from 0.98 to 0.96 and 36% from 0.98 to

0.94 is not matched with any of the compounds. This result might suggest some antagonism between reducing the water activity and the use of these antifungal compounds might occur.

This effect was not observed *in situ* this might have been caused by *F. graminearum* radial growth being stimulated on the YES containing the compounds especially at lower water activities were growth is already limited. While visible growth in a grain monolayer occurs in the intergranular spaces with the initial grains being invaded radial growth occurs before the surrounding grains become infected. This radial growth is not easy to assess and is very dependable of the infection capacity. Moreover the growth pattern of the aerial mycelia also influences radial distribution. Moreover, *F. graminearum* was not able to grow at 0.94 a_W in the presence of any compound, this might be due to the incapacity to infect wheat irradiated grain in this conditions. The growth at this water activity was already reduced and the compound was the hurdle that completely inhibited growth.

As with growth the initial assessment of *Tri5* gene expression demonstrated that of the 5 strains tested, even though the differences were small, the strain I_3 L1-2/2D displayed higher levels of expression.

Tri5 gene expression was not directly correlated with growth rate either *in vitro* or *in situ* in the absence of anti-fungal compounds. However, there was a stimulation of *Tri5* gene expression (x 7 fold) with intermediate antifungal compounds *in vitro* with the thyme essential oil, especially at the lower water activity of 0.94, although effect on growth was minimal. BHA was the only compound where *in vitro* results show similarity with the *in situ* expression. In both cases this compound stimulated the expression of *Tri5* in high water activity conditions while at intermediate conditions the expression was reduced by the presence of BHA. Nevertheless even in this case and with the presence of ethanol the control displayed a stable expression of *Tri5 in vitro* but *in situ* the expression appeared to be stimulated when water stress was imposed. The results obtained from the *in vitro* control samples where concordant with what had been previously reported by Marín *et al.* (2010) and by Schmidt-Heydt *et al.* (2011) although this study did not consider antifungal compounds but only the

effect of a_W x temperature on *Tri5* expression *in vitro*. The *in situ* results appear to be the opposite of those obtained for growth rate, with higher expression of *Tri5* found occurring under water stress. These results might be the result of *F. graminearum* responding to both environmental and chemical stress by increasing secondary metabolite production to try and survive and compete more effectively in the grain environment. Results from field experiments with ripening wheat between anthesis and harvest have shown that *Tri5* expression is important and essential for colonisation of wheat spikelets (Maier *et al.,* 2006). *F. graminearum* growth in wheat is characterized by aerial mycelium in the intergranular spaces. The higher expression of *Tri5* can be the result of a wider infection front and a possible toxicological response to adversity, demonstrating the possible role of trichothecenes in wheat infection (Hallen-Adams *et al.*, 2010; Alexander *et al.*, 2009).

The *in situ* results obtained for trichothecenes type B production confirm the risk of using sub-lethal doses of antifungal compounds as they may cause a stimulation of mycotoxin contamination. The increased expression levels of *Tri5* in the presence of Prochloraz and BHA *in situ* were supported by the high amounts of Trichothecenes type b including DON in the wheat irradiated grain. While there is a lot of interest in the use of essential oils to control mycotoxins in different crops it is clear that intermediate concentrations may not be effective at mycotoxin control. Thus *Tri5* expression in response to thyme essential oil showed that regardless of a_W levels, there was a significant stimulation. This was similar to the increase in DON observed in the analytical analysis. This confirms previous work on *P. verrucosum* and ochratoxin A production and *otapks*Pv expression correlations previously (Schmidt-Heydt *et al.*, 2007). This study was the first to show that in the presence of sub-optimal concentrations of propionate preservatives both *otapks*Pv and ochratoxin A was significantly stimulated at different a_W levels while growth was actually inhibited. The results in the present study support these findings for *F. graminearum* and *Tri5* gene expression. Previously Magan *et al.* (2002) showed that lowered concentrations of different fungicides could stimulate DON production. However, this study did not consider the molecular aspects. There has not been any previous information on the effect of intermediate concentrations of antifungal compounds on a family of

mycotoxins such as the trichothecene type B group. This study has shown that there are very interesting effects on the relative ratios of different mycotoxins in this group produced depending on a_W x antifungal compound used. Thus 3 AcDON levels appeared to increase in the presence of thyme essential oil and overall the acetylated forms were found in higher amounts when *F. graminearum* was grown in the presence of prochloraz. Moreover the acetylated forms of DON represented a higher percentage of the total trichothecenes type B since they are precursors of DON. The increased production of 3 AcDON over 15 AcDON can be due to modulation of *Tri8* gene expression. The amounts of trichothecene type B detected at 0.94 were constant during the 20 days for all treatments and controls and they perhaps indicate previous wheat grain contamination. Even though at this water activity conditions *Tri5* expression was stimulated there was no comparative toxin increase. This result demonstrates the complexity behind trichothecene biosynthetic pathway regulation.

These results show how critical temperature, water activity and the application of antifungals in pre-harvesting and post-harvesting conditions are in the colonization by *F. graminearum*. These conditions can represent a shift in the causal agent of FHB and severity in important wheat producing regions. *F. culmorum* is the major cause of FHB in regions like the UK but it is being outcompeted by *F. graminearum* (Magan *et al.*, 2011). The production profile of DON and the growth rate of *F. graminearum* are higher at 25ºC compared to the 15ºC under the same water stress conditions for *F. culmorum* reported by Hope and Magan (2003).

This study suggests that it is imperative to use effective concentrations of antifungal compounds to completely control *F. graminearum* growth. None of the compounds was capable of controlling trichothecenes and *Tri5* expression in all conditions tested. Moreover, the application of sub lethal concentrations may result in a relatively higher risk of mycotoxin production stimulation, leading to core contamination of cereal grain.

5 The impact of anti-fungal compounds and environmental stress on *Penicillium verrucosum*, ochratoxin production and expression of *otapks*Pv gene using RT-qPCR.

5.1 Introduction

Penicillium verrucosum is an important mycotoxigenic filamentous fungus, especially in northern Europe where the climate is cooler. It is an important ochratoxin A (OTA) producer and is predominantly responsible for OTA contamination of cereals and their sub-products (Lund and Frisvad, 2003; Schmidt-Heydt *et al.*, 2007). Contamination of cereals with OTA predominantly occurs post-harvest during storage, and is especially recurrent in regions were harvesting is done under cooler moist conditions as in northern Europe (Lund and Frisvad, 2003; Aldred *et al.*, 2008). Inefficiently dried grain can lead to pockets of *P. verrucosum* establishment. Moreover inefficient incorporation of preservatives can lead to under-treated pockets that can not only lead to growth but also to stimulation of OTA production (Arroyo *et al.*, 2005; Shmidt-Heydt *et al.*, 2007). Studies by Schmidt-Heydt *et al.* (2009) showed that intermediate concentrations of propionate preservatives can inhibit growth but stimulate ochratoxin A production by *P. verrucosum* and this was confirmed by examining the expression of the *otapks*Pv gene which was correlated with ochratoxin A production.

OTA is of importance in terms of human health concerns. Not only because it is one of the most common naturally occurring mycotoxin, but because it contaminates a wide range of commodities including cereal, coffee, wine, spices, dried fruits, beer, grape juice as well as animal products. It is nephrotoxic and has potentially carcinogenic properties and is an accumulative toxin with slow excretion (Ringot *et al.*, 2006; Shmidt-Heydt *et al.*, 2007; Marin-Kuan *et al.*, 2008; Abbas *et al.*, 2009).

European legislation limits the maximum allowed OTA contamination of unprocessed cereals to 5 µg per Kg and a lower 3 µg per Kg for cereals or cereal products destined for direct human consumption. The European Food Safety Agency (EFSA) has

established a Tolerable Weekly Intake (TWI) for OTA of 120 ng/kg body weight, EFSA estimate the average dietary intake of OTA in Europe to be lower than the TWI.

The OTA biosynthetic pathway has not yet been fully revealed. Nevertheless, the OTA molecule is composed of a polyketide dihydroisocoumarin moiety linked via an amide bound to phenylalanine. A non-ribosomal peptide synthetase is most likely to be involved in this step (Abbas, *et al.*, 2009). Recently, a gene encoding a non-ribosomal peptide synthetase (NRPS) has been described as part of OTA biosynthetic pathway in *Aspergillus carbonarius*. The knockdown of this gene produced an interesting effect of increasing ochratoxin β, the dechloro analog of ochratoxin α which together with OTA was absent. This would suggest that the phenylalanine bound to the polyketide dihydroisocoumarin would precede the chlorination step of ochratoxin β (Gallo *et al.*, 2012). Labelling experiments showed that the phenylalanine moiety results from the shikimate pathway and the polyketide dihydroisocoumarin moiety arises from the pentaketide pathway (Ringot *et al.*, 2006). Furthermore the first step of the pentaketide pathway involves a polyketide synthase and is encoded in *P. verrucosum* by *otapks*Pv gene.

Environmental factors have a profound effect on production of mycotoxins such as OTA. The most important factors controlling fungal growth in foodstuffs are temperature, water activity (a_W) and pH or the intergranular gas composition depending on the food matrix in question (Magan, 2007; Magan and Aldred, 2007; Magan *et al.*, 2010). There is interest in examining the impact that interactions between anti-fungals (butylhydroxyanisole, Prochloraz and thyme essential oil) and water availability have on growth and *otapks*Pv gene expression and phenotypic mycotoxin production.

The aims of this study were (a) assess the influence of intermediate concentrations of an essential oil, fungicides and anti-oxidant on growth of *P. verrucosum* under different a_W x temperature conditions, (b) evaluate the impact of environmental factors and the presence or absence of anti-fungal compounds have on the expression of *otapks*Pv gene and (c) the effect that these compounds at intermediate concentrations have on OTA production.

5.2 Materials and Methods

5.2.1 *Penicillium verrucosum*

Penicillium verrucosum isolate OTA11 is a known OTA producer. The cultures were maintained on Malt Extract Agar (MEA) media (OXOID, malt extract, 30; mycological peptone, 5; agar, 15 g/L).

5.2.2 Ochratoxin expression growth media

OTA expression was induced by subculturing *P. verrucosum* into Yeast Extract Sucrose (YES) medium which is a mycotoxin inducing medium (20.0 g/L Yeast extract, 150.0 g/L Sucrose, 15 g/L Agar) (Davis, *et al.* 1966).

Around 20 ml of YES medium were poured into each 90mm plate, and then after cooling, a sterile 90 mm disc of cellophane would be placed aseptically into each plate in order to collect the entire mycelium.

5.2.3 Water activity adjustment

The a_W of YES medium was adjusted to 0.98, 0.95 and 0.93. The final a_W of the media was measured using an Aqualab® 3TE (Aqualab, USA) water activity analyser. The calibration was carried out as previously described in Chapter 4, Section 4.2.3

5.2.4 Inoculation and incubation

Cultures of the *P. verrucosum* OTA11 were prepared on MEA and incubated at 25°C for 10 days. Spore suspensions were prepared has described in Chapter 4, Section 4.2.4. and adjusted by dilution to 10^6 spore's ml^{-1}.

Petri plates with glycerol modified YES were inoculated with 10µl of the spore suspension and incubated at 20, 25 and 30ºC. The experiments were carried out with three replicates per treatment.

5.2.5 Growth assessment

For each replicate the radial growth was measured in two directions at right angles to each other with a ruler. The growth rate was calculated based on the slope of the linear region of the growth curve for each replicate.

5.2.6 Preparation of stock solutions of the antifungal compounds

Stock solutions of butylated hydroxyanisole (BHA), prochloraz and thyme essential oil were prepared in sterile distilled water. The solutions were then filter-sterilised through a sterile 0.2 µm Millipore filter (Minisart, Sartorius) into sterile containers. When higher concentrations of the compounds were required or when the solubility of the compounds did not allow the preparation of water solutions, stock solutions were prepared in 50% absolute ethanol (HPLC Grade). These were incorporated into the agar media or added to the wheat irradiated grain to take account of the target a_W levels for the treatments. Suboptimal concentrations of propionate preservatives lead to an increase in ochratoxin production. A summary of the Suboptimal conditions used is shown at Table 5.1.

Table 5.1 - Treatment conditions used during this chapter.

Compound	Final concentration	Glycerol modified a_W	Temperature
Control	-	0.98	15ºC 20ºC 25ºC
Control + Ethanol	1 ppm	0.96	25ºC
BHA	150 ppm + 1 ppm Ethanol	0.94	
Prochloraz	0.2 ppm		
Oil of thyme	0.2 ppm		

5.2.7 Ochratoxin A analysis

In summary, after collection of the cellophane with the mycelium, agar plugs of 4mm were taken using a cork borer and placed into 2 ml Safelock tubes and weighed.

OTA was extracted by adding 1 ml of HPLC grade methanol and shaking for 1 hour. The supernatant extracts were filter directly into amber HPLC vial (Agilent technologies, UK), they were kept at 4ºC until analysis.

The HPLC vials were transferred to an Agilent 1200 series system, composed by a degasser, a binary pump, an automatic sampler and a fluorescence detector (Agilent technologies, UK) ($\lambda_{excitation}$ 333 nm and $\lambda_{emission}$ 460nm). The samples were separated using a C_{18} Phenomenex Luna column (150 mm x 4.6 mm, 5 µm) (Phenomenex, UK), preceded by a guard cartridge (3mm x 4.6mm) containing the same material. The samples were run using isocratic elution. Mobile phase composition was acetonitrile: water: acetic acid (57:41:2 v/v/v). The flow rate was 1 ml/min. The run time was 12 min and OTA was detected at about 5.25 min.

The area underneath the peak presenting the same retention time than the OTA standard was measured and compared against a standard calibration curve built with the OTA standard in order to calculate the mycotoxin concentration.

5.2.8 Total RNA extraction

The fungal biomass was quickly frozen in liquid nitrogen and stored at -80ºC until RNA extraction could be carried out.

To increase efficiency and decrease the variability on the RNA yields, an automatic total RNA extraction and purification system was used (see Chapter 3).

5.2.9 Reverse transcriptase PCR

Penicillium verrucosum total RNA was reversed transcripted using the previously described method in Chapter 4, Section 4.2.8.

5.2.10Gene expression

Polyketide synthase is involved in the first step of the pentaketide pathway in the biosynthetic pathway of OTA. *OTA polyketide synthase (otapksPv)* gene expression has been previously corelated with the production of OTA for this reason the gene expression of *otapksPv* was accessed by RT-qPCR using the set of primers on Table 5.1

Table 5.1 - *Penicillium verrucosum OTA polyketide synthase (otapksPv)* gene primers, gene accession number DQ789993.1

Name	Sequence	Position
Otapks-SYBR1_For	5'-TTG CGA ATC AGG GTC CAA GTA	894
Otapks-SYBR1_Rev	5' – CGA GCA TCG AAA GCA AAA ACA	944

This primer pair produces a 50bp fragment.

It was also amplified a different set of amplicons, targeting one reference gene in order to normalise the expression levels between the different experiments and samples. The pair of primers in Table 5.2 were used no normalise the gene expression.

Table 5.2 - *Penicillium verrucosum β-tubulin* gene primers.

Name	Sequence
PV-bentaq_for	5' – CTA GGC CAG CGG TGA CAA GT
PV-bentaq_rev	5' – CAT GGT ACC GGG CTC CAA

Standard curves were generated as previously described in section 4.2.10. In summary, a total RNA pool was created using RNA extracted from different *P. verrucosum* samples. From this RNA pool 2 µg of total RNA were reversibly transcripted into cDNA.

A range of 10 fold diluted cDNA was generated from this pool and RT-qPCR was carried out using these samples.

The standard curves generated for *P. verrucosum otapks*Pv and *β-tubulin* genes is presented in Figure 5.1, were evaluated as previously described in section 4.2.10.

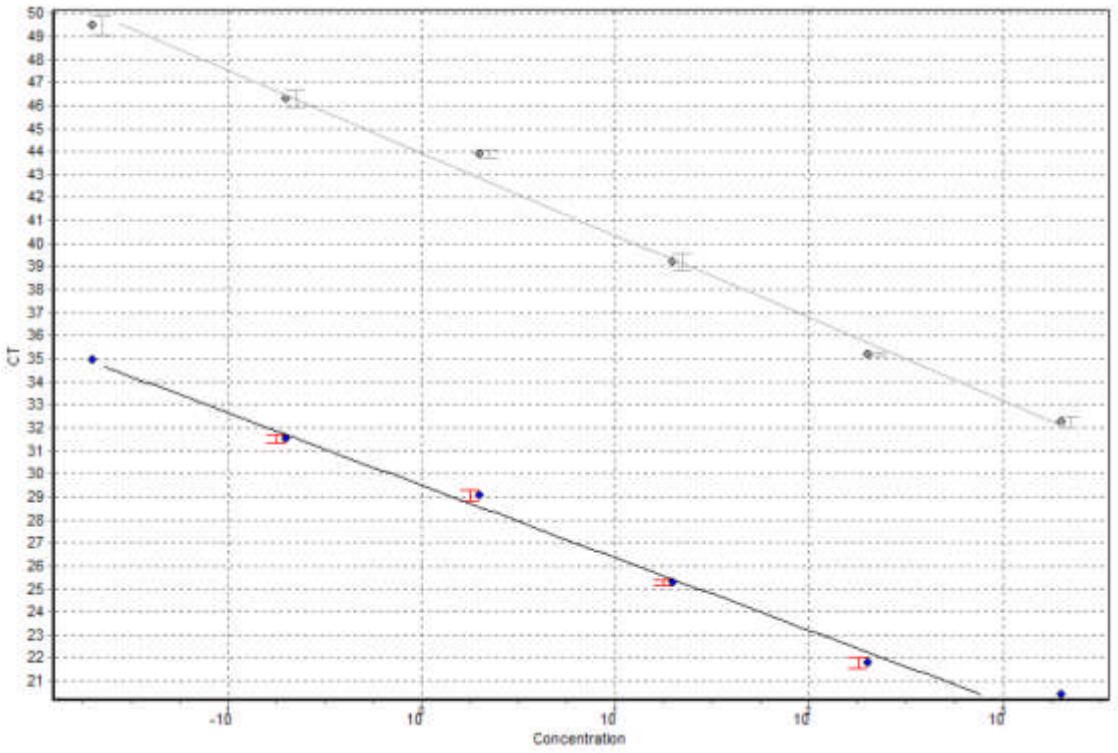

Figure 5.1 - Standard curves used to calculate amplification efficiency for each gene (*otapks*Pv and *β-tubulin* as reference gene) for relative quantification using the Pfaffl method. Light grey line represents *otapks*Pv standard curve while darker line represents *β-tubulin* standard curve. The vertical lines represent the mean standard error. The efficiency of *otapks*Pv was of 1.08 with an R value of 0.99139 while the efficiency of *β-tubulin* was 0.90 with an R value of 0.99351.

Gene expression quantification was carried out as described in section 4.2.10 using the bioinformatic Q-gene application and applying Equation 4.1 to the CT results obtained from the Rotorgene Q (Qiagen) software. Further melt curve analysis was used to assess the RT-qPCR reaction quality.

5.3 Results

5.3.1 Effect of water activity x temperature on *Penicillium verrucosum* growth

Initially *P. verrucosum* growth was assessed to determine the impact of environmental parameters (temperature x a_W: Figure 5.2). This showed that growth was fastest at 0.98 a_W and 20-25^{o}C. Growth at 15ºC and 0.94 a_W was more marginal for growth.

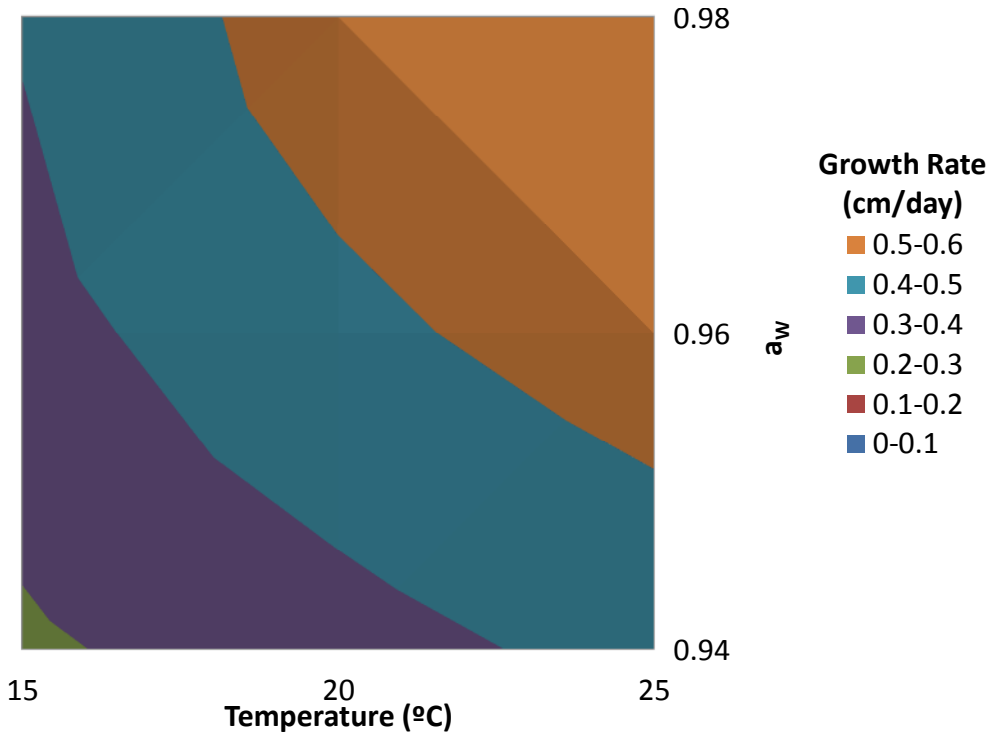

Figure 5.2 - Contour map of the effect of water activity x temperature on the growth of *P. verrucosum* on YES medium. Colours indicate the different growth rate regions.

Subsequently, the antioxidant (BHA), essential oil (oil of thyme) and fungicide (Prochloraz) were tested at a sub-lethal concentration in order to evaluate the impact of each compound (Figure 5.3). BHA is not soluble in water, it was solubilized in ethanol and the ethanol effect was also evaluated.

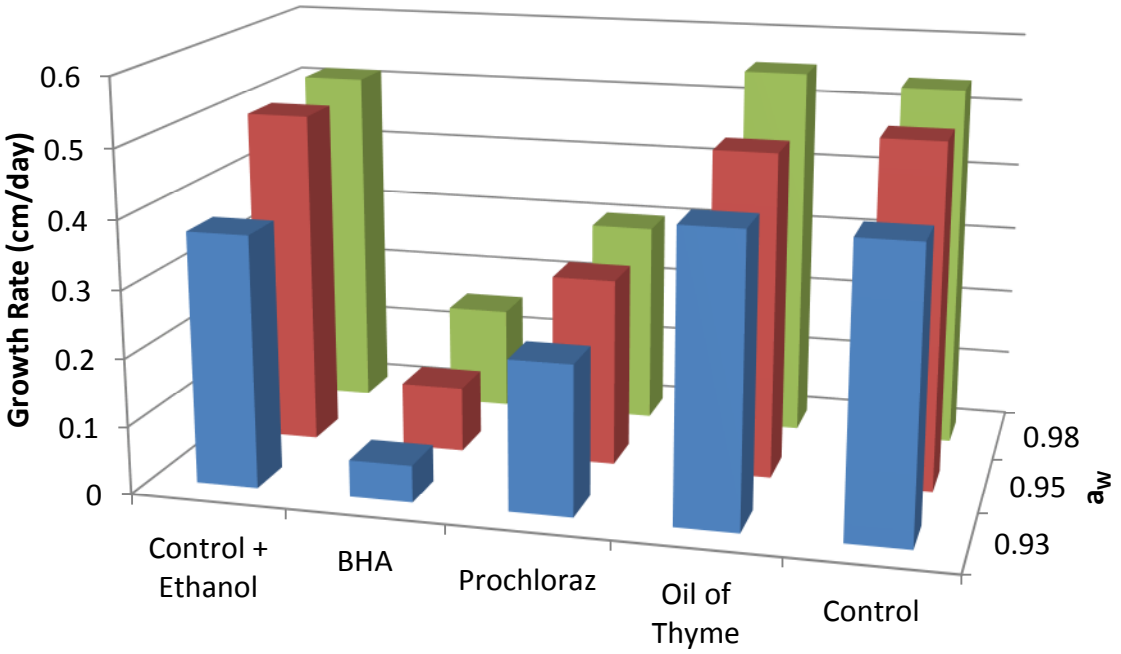

Figure 5.3 - Influence of anti-fungal compounds on growth of *P. verrucosum* under different water stress conditions at 25ºC.

5.3.2 Effects of environmental conditions on OTA production

The production of OTA was measured by HPLC using the method described above. The results for the temporal effect of environmental factors showed an increased amount of OTA production at 25ºC and intermediate a_W levels (Figure 5.4). Interestingly at intermediate a_W levels some stimulation of OTA was observed (e.g. 0.96 a_W).

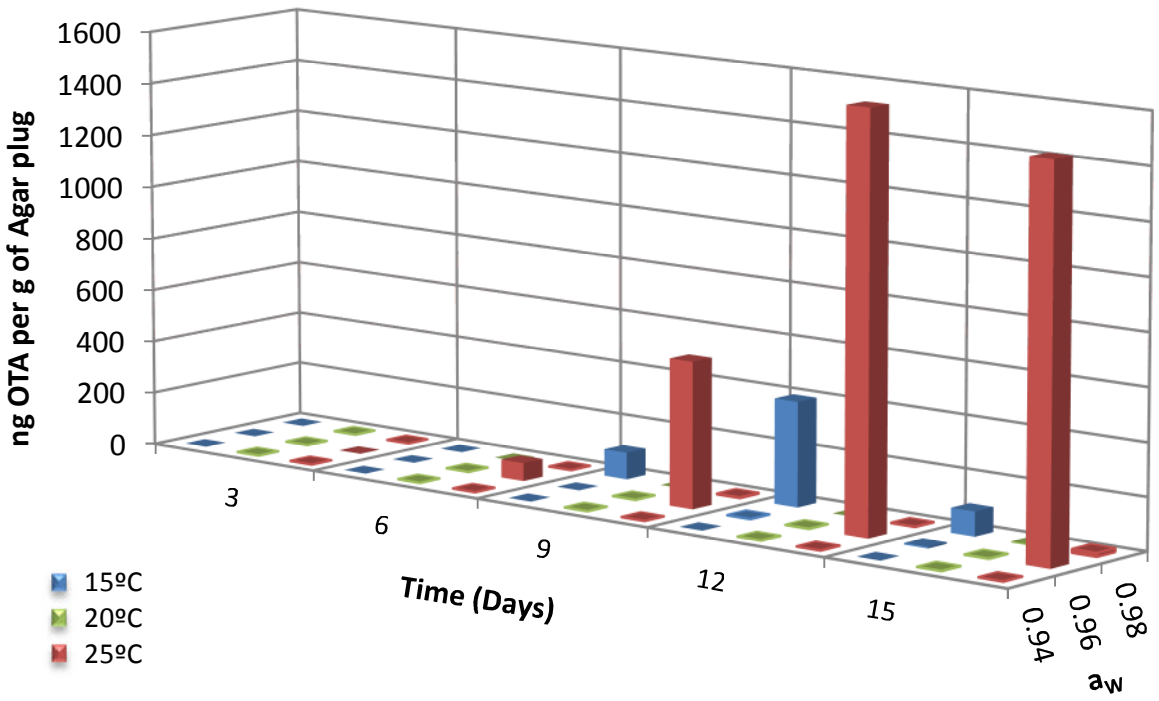

Figure 5.4 - *Penicillium verrucosum* OTA production in relation to different water stress conditions versus temperature conditions.

Since a higher production of OTA occurred at 25ºC, the impact of the different compounds was evaluated at this temperature. Due to the use of ethanol to solubilise BHA, the effect of ethanol alone was also evaluated serving as control to the impact of BHA on the production of OTA (Figure 5.5).

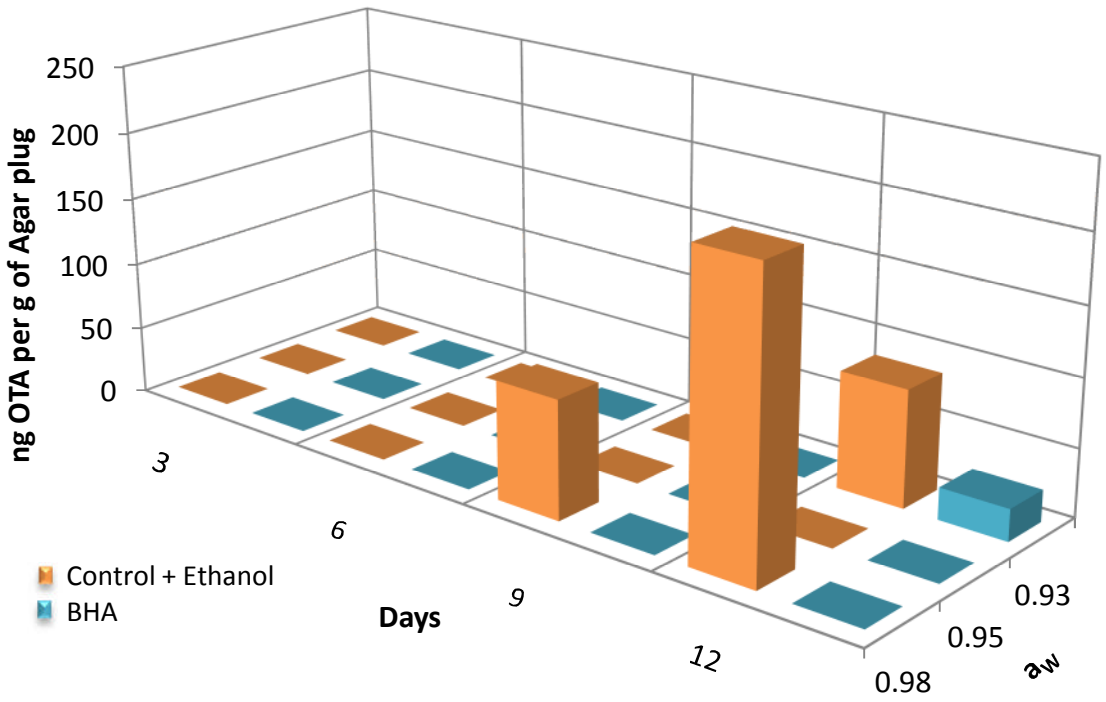

Figure 5.5 - Influence of BHA on OTA production by *P. verrucosum* under different water stress conditions at 25ºC.

The antioxidant BHA was able to reduce the amounts of OTA produced by *P. verrucosum* at all water stress conditions tested, even though it apparently is more effective at higher a_W levels. This suggests that OTA production is not only activated as an oxidative stress, as recently suggested in the literature.

The effect of prochloraz on OTA production followed the same pattern as for BHA (Figure 5.6). However, the essential oil of thyme induced an increase in OTA production at intermediate water stress conditions.

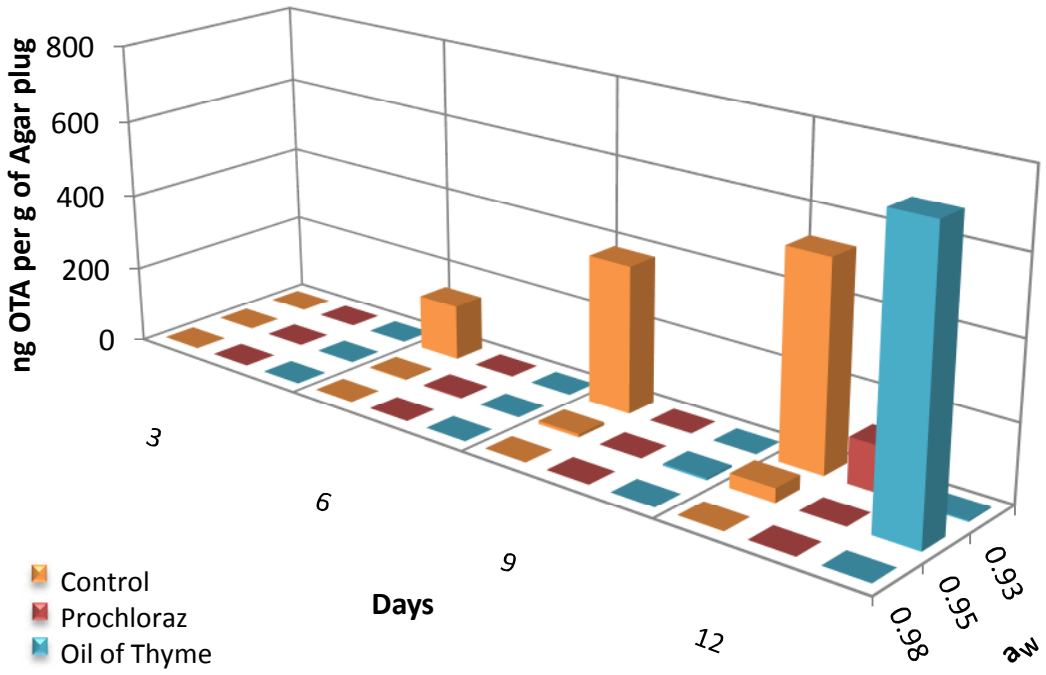

Figure 5.6 - Influence of prochloraz and oil of thyme, on OTA production by *P. verrucosum* under different water stress conditions at 25ºC.

5.3.3 *P. verrucosum otapks*Pv gene expression

*P. verrucosum otapks*Pv gene expression was quantified at day 9 using RT-qPCR. The impact of BHA under different a_W conditions is presented at Figure 5.7. BHA down regulated *otapks*Pv expression at both 0.98 and 0.93 a_W levels water stress conditions, with higher impact at higher a_W levels tested.

The impact of Prochloraz and the essential oil of thyme under different water stress conditions, on *otapks*Pv gene expression are shown in Figure 5.8. Sub-lethal doses of these compounds had contrasting effects on *otapks*Pv gene expression. Prochloraz upregulated the expression of *otapks*Pv at higher a_W conditions of 0.96 and 0.95. On the other hand the essential oil of thyme down regulated *otapks*Pv gene expression at all water stress conditions tested. One further interesting result comes from the control samples were the presence or absence of ethanol shifted the *otapks*Pv maximum gene expression from high to lower water activity conditions, respectively.

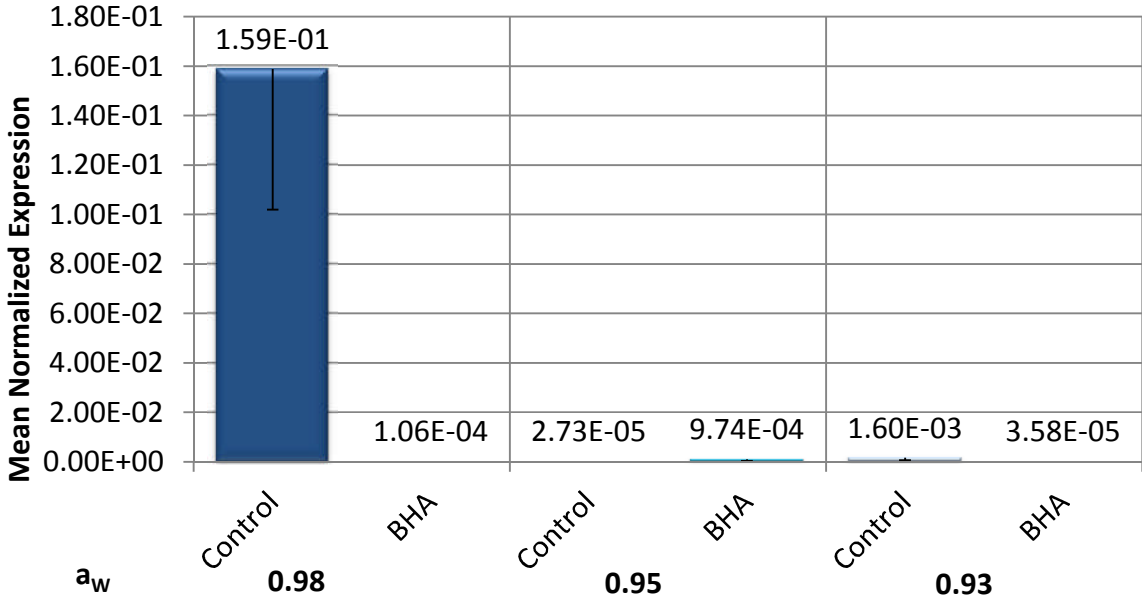

Figure 5.7 - Influence of water stress conditions and BHA on *otapks*Pv gene expression at different a_W levels. Darker blue colour represents 0.98 water activity while lighter colours represent 0.93 water activity. The middle bars represent 0.95 water activity conditions. Vertical lines indicate negative Standard error of the mean.

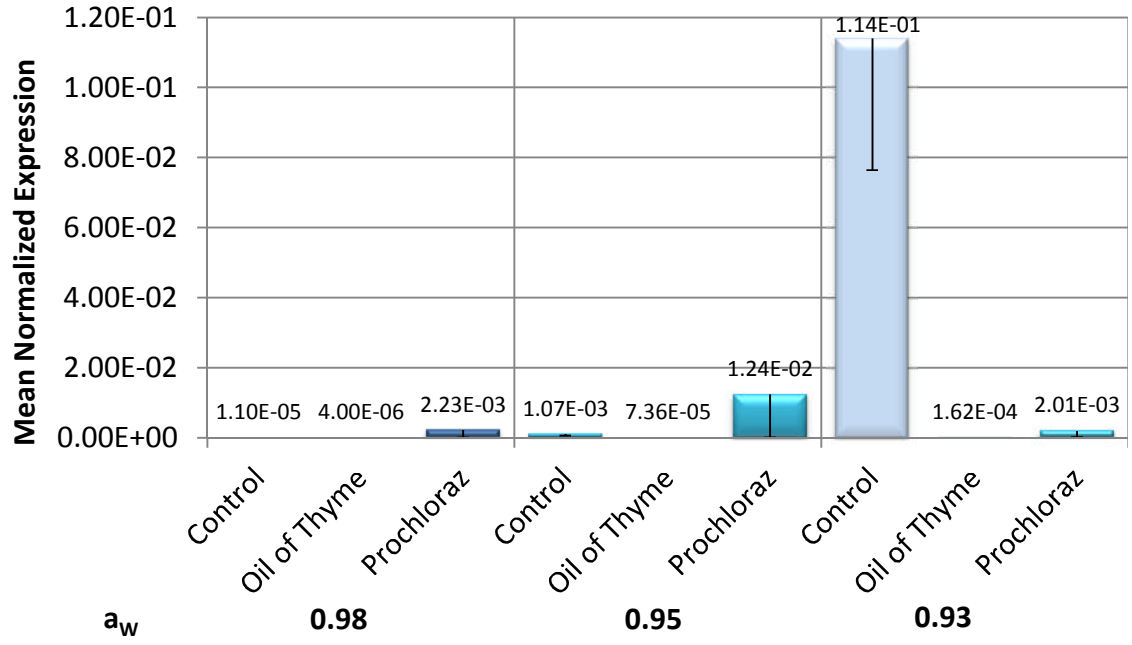

Figure 5.8 - *otapks*Pv gene expression in relation to antifungal compounds at three different a_W levels. Darker blue colour represents 0.98 water activity while lighter colours represent 0.93 water activity. The middle bars represent 0.95 water activity conditions. Vertical lines indicate negative standard error of the mean.

5.4 Discussion

Penicillium verrucosum OTA11 growth was influenced by a_W x temperature interactions. The growth was reduced at 15^oC by about 50% when compared to that at 25^oC, with growth significantly reduced at 0.94 a_W. Interestingly at 25ºC the maximum growth rate was under intermediate a_W stress (0.96 a_W), while at lower temperatures this occurred at >0.98 a_W. *P. verrucosum* limit for any potential growth or OTA production at 25ºC has been estimated for wheat based medium to be 0.80 a_W, with optimal growth and OTA production conditions within the range used in this study (Cairns-Fuller *et al.*, 2005). Overall the growth rate results presented in this study were higher than that reported by Cairns-Fuller *et al.* (2005), nevertheless the overall trend of effect of a_W x temperature effects on growth were comparable.

Results in Chapter 2 suggested that even low amounts of mixtures of preservatives could severely hinder *P. verrucosum* growth. Nevertheless, that is most likely due to the poor nutrient conditions that the growth boundaries study was carried out under. *F. graminearum* I_3 L1-2/2D was capable of growing under all mixed preservative conditions tested. Nevertheless, in the presence of 0.1 ppm of prochloraz *F. graminearum* I_3 L1-2/2D growth was reduced by up to 44%, similar to the reduction observed for *P. verrucosum* OTA11. Moreover *P. verrucosum* was capable of growing even in the presence of 150 ppm of BHA. Under this condition grow was significantly affected. In contrast, 0.22 ppm of essential thyme oil appeared to slightly stimulate growth. This effect was also detected for *A. westerdijkiae* where Aldred *et al.* (2008) reported an increase in growth and OTA production at 50 ppm of essential oil of thyme. In their study a higher concentration range was used and this makes direct comparisons more difficult.

Interestingly by using intermediate concentrations of the essential oil there was a difference in the effect on OTA production. There was a stimulation of OTA production observed when compared with the other anti-fungal treatments used. This was especially observed at intermediate a_W levels. Previously, Schmidt-Heydt *et al.* (2010) described the circadian regulation of OTA biosynthesis with both *otapks*Pv gene expression and OTA production showing a cycle of an increase in amounts under

light/dark conditions. In the present study a similar effect was observed between 9th and 15th day at 15ºC and 0.98 a_W and 25ºC at 0.96 a_W. Unlike OTA phenotypic expression the biosynthetic gene *otapks*Pv was only up regulated by the presence of prochloraz at high water stress conditions and BHA at 0.95 a_W. All the other conditions tested indicated down regulation of *otapks*Pv. Interestingly even though the essential oil of thyme had the higher OTA production after 12 days the expression of *otapks*Pv was much reduced at all water stress conditions. Gene expression was measured at day 9 when OTA was still sometimes not detected at any growth condition in the presence of the antifungal compounds. Expression kinetics of *P. verrucosum otapks*Pv was previously described to have a clear increase of gene expression at the later growth phases around the 8th or 9th day after inoculation (Schmidt-Heydt *et al.*, 2007). The antifungal compounds could have delayed this increased expression phase and doing so the expression of *otapks*Pv might be deviating from the control because of the culture growth stage. Overall in the controls there was a good correlation between expression of the *otapks*Pv and OTA production not only at 9 but also at 12 days.

The antioxidant examined appeared to quite effective against different mycotoxigenic fungi. Thus BHA was able to reduce *otapks*Pv gene expression and OTA production. It was also shown to reduce *Tri5* gene expression and DON production by *F. graminearum* in almost all conditions tested (see Chapter 4). Antioxidants like resveratrol and propyl paraben have been found to also be effective against mycotoxigenic fungi and control production of mycotoxins such as aflatoxins and fumonisins. Some studies even suggest the possibility of synergism between mixtures of different antioxidants (Etcheverry *et al.*, 2002; Reynoso *et al.*, 2002; Torres *et al.*, 2003; Passone *et al.*, 2007; Aldred *et al.*, 2008) Even though their mechanism of action has not been entirely clear in the case of BHA it has been suggested that cell membrane components and the mitochondrial electron chain are the primary targets (Degré and Sylvestre, 1983; Aldunate *et al.*, 1992; Khan *et al.*, 2001).

This study demonstrates the importance of appropriate application of antifungal compounds. Even in conditions were growth was inhibited sub optimum concentrations of antifungal compounds lead to OTA production stimulation. On the other hand, it also revealed that BHA not only affected growth rate but also had the

capacity to inhibit key OTA biosynthetic gene expression and toxin accumulation. Since this antioxidant as well as other food grade antioxidants are considered safe and have GRAS status they can be a good alternative to mycotoxin control compounds.

6 The use of siRNA to inhibit the mycotoxin biosynthetic pathway in *F. graminearum* and *P. verrucosum*

6.1 Introduction

In the previous Chapters, it was shown that mycotoxigenic filamentous fungi are not only able to grow in low nutrient conditions with commonly used preservatives (Chapter 2). They can also thrive in pre and post- harvesting conditions in the presence of suboptimal conditions of antifungal compounds which can sometimes stimulate mycotoxin production by *F. graminearum* (DON) and *P. verrucosum* (OTA; Chapters 4 and 5). Appropriate use of these compounds at the right concentrations is a fundamental aspect which determines their efficiency in controlling mycotoxigenic spoilage fungi and their ability to produce mycotoxins.

Codex Alimentarius advises that control of mycotoxin contamination in the food chain should be based on effective pre-harvest GAP as part of any prevention strategy. The adoption of mitigating measures during these early stages contributes to the overall potential for success in reducing mycotoxin contamination. The use of resistant seed varieties adjusted to the environmental conditions is one of the important components. Several seed varieties have been developed through breeding selection and genetic manipulation of important phenotypic traits such as growth conditions, nutritional value, total yield, drought tolerance, disease resistance as many other characteristics important for the specific commodity. Directives define the criteria and values that new seed varieties need to possess to be marketed in the EU, resistance to harmful disease causing fungal pathogens is one of these criteria.

Mycotoxins as secondary metabolites which are produced by mycotoxigenic fungi very early during infection. This may partially provide the competitive advantage when competing with other colonising phyllosphere fungi (Magan, 2007). Any approach which can potentially disrupt the biosynthetic pathway involved in mycotoxin production could be useful in limiting the contamination with a mycotoxin and minimise exposure to them in the food and feed chains. Previous studies have demonstrated the potential of inhibiting key regulatory genes in the biosynthetic

pathways for mycotoxin production by using knockout gene experiments to inhibit both DON and OTA production by *F. graminearum* and *P. verrucosum* respectively (Hohn and Desjardins, 1992; Proctor *et al.*, 1995; Karolewiez and Geisen, 2005; O'Callaghan *et al.*, 2013). This however has only been achieved by gene deletion of key biosynthetic pathway genes such as the *Tri5* and *otapks*Pv in mycotoxigenic strains. *Tri5* and *otapks*Pv are involved in the early stages of trichothecenes and ochratoxin A biosynthetic pathways. Results from Chapter 4 and 5 as well as several previous reports have demonstrated that their regulation and their expression profoundly affect the ability for phenotypic mycotoxin production.

RNA interference (RNAi) allows transient gene knockdown in a sequence specific manner. RNA interference is initiated by both dsRNA or siRNA molecules, both are recognized by the dicer enzyme that will cleave dsRNA into siRNA molecules. Once the dsRNA has been cleaved into around 20 nucleotides siRNA molecules these double stranded RNA molecules are unwound into single strand (ssRNA) with the RNA-dependent RNA polymerase binding to the opposite end of the strand and this guide strand being incorporated into the RISC complex with an argonaute like protein (Das *et al.*, 2011). The RISC complex endonuclease activity degrades the mRNA complementary to the guide ssRNA molecule. The RISC complex can also act at the genome level by heterochromatin formation and down regulate the gene pre-transcriptionally (Holmquist and Ashley, 2006). Nakayashiki *et al.* (2006) described that amino acid sequences similar to the proteins used in siRNA could be found in *F. graminearum*. Moreover siRNA has been described in *Aspergillus, Penicillium* and *Fusarium* species where the *Tri6* gene was targeted using RNA silencing (McDonald *et al.*, 2005; Whisson *et al.*, 2005; Ullán *et al.*, 2008; Abdel-Hadi *et al.*, 2011).

Plant expressing of homologous dsRNA has been previously reported to successfully down-regulate a *F. verticillioides* reporter gene during infection of the tobacco host (Tinoco *et al.*, 2010). More recently, transgenic expression of dsRNAs in the host plant led to silencing of homologous genes in the invading and colonizing *Blumeria graminis* (Nowara *et al.*, 2010). These results indicate that filamentous fungi may possess mechanisms for taking up dsRNAs or siRNAs from the environment and that expression

of these siRNA molecules if producing the desired effect can be a mechanism used to reduce mycotoxin contamination.

The objectives of this study were to (a) determine potential siRNA for targeting *Tri5* and *otapks*Pv gene expression, (b) assess *P. verrucosum* molecular machinery for efficient siRNA gene knockdown and (c) optimization of the best siRNA concentration for inhibition of the target genes and phenotypic expression of DON and OTA.

6.2 Materials and methods

6.2.1 Fungal strains and isolates maintenance

F. graminearum isolate I_3 L1-2/2D and *P. verrucosum OTA11* previously described in Chapters 4 and 5 respectively, were used in this study.

The cultures were maintained on Malt Extract Agar (MEA) media (OXOID, malt extract, 30; mycological peptone, 5; agar, 15 g/L).

6.2.2 Inoculation and growth conditions

Cultures of the *F. graminearum* and *P. verrucosum* were prepared on MEA and incubated at 25°C for 7 days. Spore suspensions were prepared has described in Chapter 4, Section 4.2.4. and adjusted to 10^7 spore's ml^{-1} these were used for protoplast generation.

6.2.3 Protoplast generation

A spore suspension of *F. graminearum* and *P. verrucosum* were sub-cultured in 200 ml of YES broth in 500 ml conical flask. Cultures were incubated for 24 h in the dark at 25°C shaking at 200 rpm.

The mycelium was harvested by filtration through lens cleaning tissue (Whatman 105). One gram of mycelia was transferred into 20 ml of filter sterilized enzyme solution (per 20 ml: 17 ml of H_2O, 2 ml of 0.2 M $NaPO_4$ [pH 5.8], 0.4 ml of 1.0 M $CaCl_2$, 1.4 g of NaCl, 200 mg of lysing enzyme [Sigma], and 50 mg of driselase [Sigma]. Mycelia were incubated at 30°C shaking (80 rpm) for 3 h.

Protoplasts were separated from intact mycelia by passage through lens cleaning tissue (Whatman 105) into sterile 50 ml tube, and 20 ml of sterile STC buffer (1.2 M sorbitol, 10 mM $CaCl_2$, 10 mM Tris-HCl [pH7.5]) was added. Protoplasts were pelleted by low-speed centrifugation (1,000 rpm) for 5 mins. The supernatant was carefully removed, and the protoplasts washed again in 20 ml of STC and pelleted by centrifugation as previously described. The protoplasts were then resuspended in 1.0 ml of STC buffer, and their concentration was adjusted using a haemocytometer to 10^5 protoplasts ml^{-1} (Abdel-Hadi *et al.*, 2011).

6.2.4 siRNA design

Two siRNA sequences were designed using i-Score bioinformatics tool (Ichihara *et al.*, 2007). The bioinformatic tool selects all possible siRNA sequences and scores them according to their biochemical caracteristics.

The mRNA sequence of *F. graminearum Tri5* gene accession number NT086532 (Cuomo *et al.*, 2007) and *P. verrucosum otapks*Pv gene accession number DQ789993 (Schmidt-Heydt *et al.*, 2007) were selected from NCBI database. The sequences were used to design the siRNA molecules. The five higher score siRNA sequences were selected.

To avoid off target effects the selected siRNA sequences were challenged using the nucleotide alignment search tool (blastn) against all reference sequences available and later against all filamentous fungi sequences (Altschul *et al.*, 1990). Sequences with high similiarity to other genes were excluded. The top two siRNA sequences were then selected.

These siRNA were named as Tri5_Ia and Tri5_Ib designed to target *F. graminearum Tri5* gene and otapks-Ia, otapks-Ib designed to target *P. verrucosum otapks*Pv gene (Table 6.1). The designed siRNA oligonucleotides were obtained from ambion custom silencer® select (Life technologies). Silencer® Negative Control (Life technologies) with no sequence homology and labelled with Cy3™ was used to assess transfection efficiency and influence on gene expression and toxin production.

Table 6.1 - Details of siRNA sequences used in this study

siRNA	Sequence
Otapks_Ia	Sense: CGACUACAAGGAAAUAUAUUU Antisense: AUAUAUUUCCUUGUAGUCGAU
Otapks_Ib	Sense: GGAUCACGAAGAAAUAUGAUU Antisense: UCAUAUUUCUUCGUGAUCCUU
Tri5_Ia	Sense: GGACCUUUCUGCUCAUUGA Antisense: UCAAUGAGCAGAAAGGUCCAA
Tri5_Ib	Sense: GUAGCACUAUGGACUGUAA Antisense: UUACAGUCCAUAGUGCUACGG

6.2.5 siRNA transfection

All siRNAs were resuspended in RNase free water at a final concentration of 5, 10, 25, 50 and 100 nM. In a sterile 1.5 mL micro centrifuge tubes, 10 µL of each siRNA was mixed with 1 µL of Lipofectamine™ RNAiMAX (Invitrogen Life Technologies, UK) and allowed to stand for 15 min at 20 °C. 19 µL of protoplasts (1×10^3) were added and mixed gently. The tubes were incubated at 20°C for 24 h to allow transfection to proceed. The efficiency of the transfection protocol was evaluated using an Accuri C6 flow cytometer (BD biosciences). Fluorescence difference between 0nM siRNA control and the Cy3™ labelled negative control with and without the presence of transfection reagent permitted the identification of protoplasts by their fluorescence levels.

Protoplast regeneration was enhanced by adding 70 µL of YES broth with 1.2 M of sorbitol to the transfection mixture and incubation at 25ºC for another 24 h of this suspension. The entire 100 µL of protoplast suspension were spread in wheat agar medium (20 g per l of milled wheat and 20 g per l of technical agar) and incubated at 25 °C in the dark. All experiments were carried out using three biological replicates.

6.2.6 siRNA effect on mycotoxin production

(a) Ochratoxin A

OTA was extracted by adding 1 ml of HPLC grade methanol per g of sample and shaking for 1 hour. The supernatant extracts were filtered into 20 ml tubes and allowed to dry at room temperature.

The extracts were resuspended using 1 ml of HPLC grade methanol and filtered into amber HPLC vial (Agilent technologies, UK), they were kept at 4ºC until analysis as described in Chapter 5. Section 5.2.7.

(b) Trichothecene type B

Trichothecenes were extracted by adding a mixture of acetonitrile:water (84:16 v/v) to the pre weighed sample on a ratio of 4 parts extraction mixture to 1 of sample (v/w). This mixture was then shaken overnight. The supernatant extracts were passed through a cleanup cartridge composed of a disc of filter paper and 500 mg of alumina: activated carbon (20:1 w/w). The cartridge was then washed with a mixture of acetonitrile:methanol:water (80:5:15 v/v/v). The combined eluate was freeze dried and re-suspended in a solution of water: acetonitrile (9:1 v/v). This solution was filtered into amber HPLC vial (Agilent technologies, UK), and kept at 4ºC until analysis.

The HPLC vials were transferred to an Agilent 1200 series system, composed by a degasser, a quaternary pump, an automatic sampler and a UV diode-array detector (Agilent technologies, UK). Chromatograms were generated by recording the signal at 218.4 nm. The samples were separated using a C_{18} Agilent Poroshell 120 column (150 mm x 4.6 mm, 2.7 µm) (Agilent, UK), preceded by a guard column. The samples were run at a flow rate of 1 ml min^{-1} using the following gradient elution containing acetonitrile and water: 0-1 min isocratic with 5% acetonitrile; then a linear increase from 5% to 12% acetonitrile from 1-7 min; then a linear increase from 12% to 30% acetonitrile from 7-8 min; then from a linear increase from 30% to 40% acetonitrile from 8-10 min; then a isocratic step at 40% acetonitrile from 10-11 min; an linear increase from 40% to 99% acetonitrile from 11-12 min, followed by an isocratic

washout step for 5 min and a reconditioning step to shift back to acetonitrile: water (5: 95; v/v). NIV was detected at about 5.32 min, DON at about 8.13 min, 3AcDON at about 11.24 min and 15 AcDON at about 11.34 min.

The results obtained at the samples were compared against a standard calibration curve of trichothecenes type B.

6.2.7 siRNA effect on gene expression

(a) Total RNA extraction

As described in Chapter 3

(b) Reverse transcriptase PCR

As described in Chapter 4, Section 4.2.9.

(c) Gene expression

As described in Chapter 4, Section 4.2.10 for *Tri5* and Chapter 5, Section 5.2.10 for *otapks*Pv gene expression.

6.3 Results

6.3.1 Transfection efficiency

The use of a labelled siRNA oligonucleotide allowed the evaluation of transfection efficiency. Graphical results are showed in Figure 6.1 for *P. verrucosum* and Figure 6.2 for *F. graminearum*. Around 81% of *P. verrucosum* and 84% of *F. graminearum* protoplasts had increase fluorescence with the use of the transfection reagent Lipofectamine™ RNAiMAX.

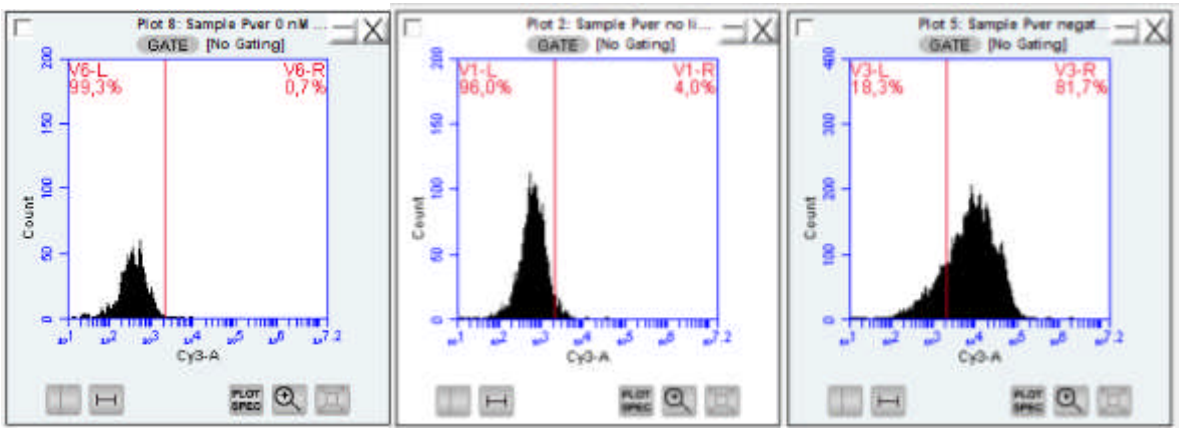

Figure 6.1 - Graphical representation of the detected protoplasts and their levels of fluorescence. The left plot represents *P. verrucosum* protoplast transfected with no siRNA. The middle plot presents *P. verrucosum* protoplast transfected with no transfection reagent. The right plot presents *P. verrucosum* protoplast transfected with Cy3™ labelled negative control siRNA.

Moreover in the absence of the transfection reagent *P. verrucosum* still registered and increase of 3% of the number of protoplast with fluorescence. The gate for evaluating the different populations of protoplast was set a 2.069 fluorescence of the Cy3 channel.

To further follow the siRNA oligonucleotides delivery, con-focal microscopy images were obtained 24 and 48h after transfection of *P. verrucosum* (Figure 6.3) and *F. graminearum* (Figure 6.4).

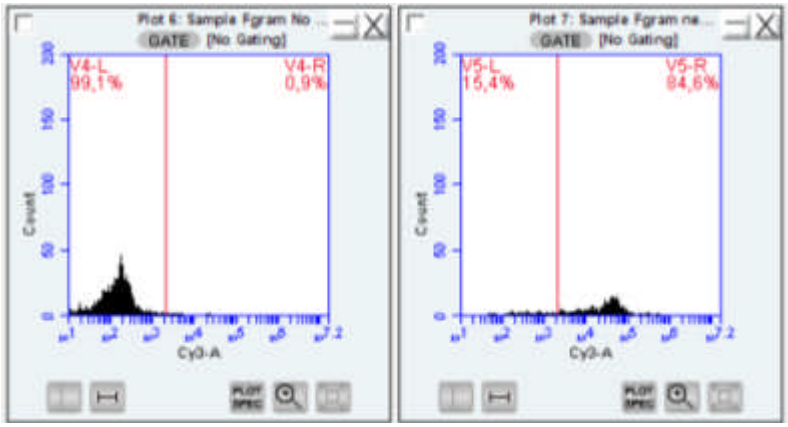

Figure 6.2 - Graphical representation of the detected protoplasts and their levels of fluorescence. The left plot represents *F. graminearum* protoplast transfected with no transfection reagent. The left plot presents *F. graminearum* protoplast transfected with Cy3™ labelled negative control siRNA.

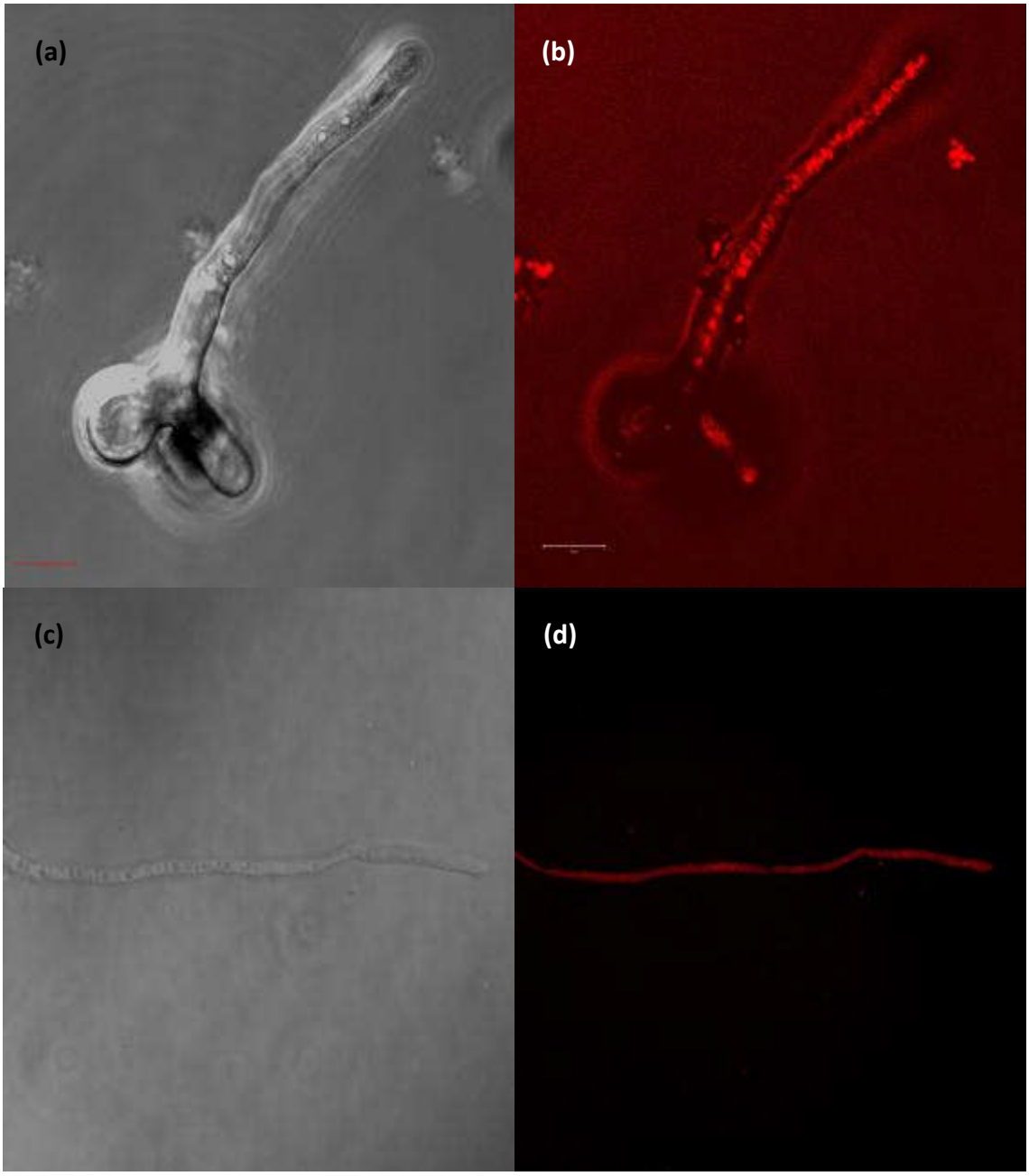

Figure 6.3 - Confocal images of *P. verrucosum* protoplasts after 24h (a, b) and 48h (c, d). Images obtained by confocal differential interference contrast (DIC) (a, c) and fluorescence microscopy (b, d) of *P. verrucosum* mycelium transfected with Cy3™ labelled negative control siRNA.

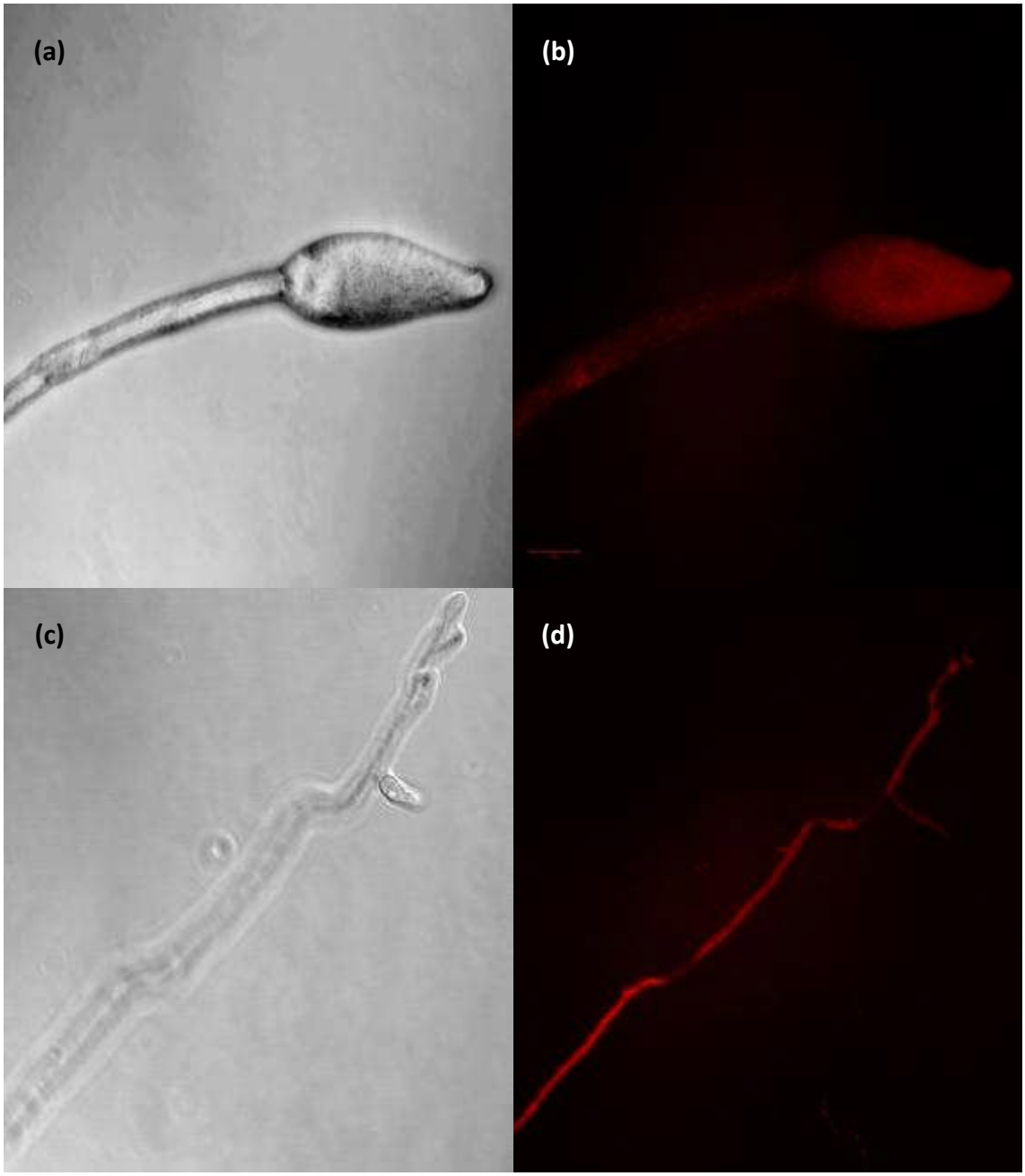

Figure 6.4 - Confocal images of *F. graminearum* protoplasts after 24 h (a, b) and 48 h (c, d). Images obtained by confocal differential interference contrast (DIC) (a, c) and fluorescence microscopy (b, d) of *F. graminearum* mycelium transfected with Cy3™ labelled negative control siRNA.

6.3.2 Effect of small interfering RNA on mycotoxin production

Ochratoxin A production by *P. verrucosum* was evaluated after 6 and 15 days. The impact of varying siRNA concentration is shown in Figure 6.5. Trichothecenes type B toxins were below the method limit of detection in all samples including the control samples.

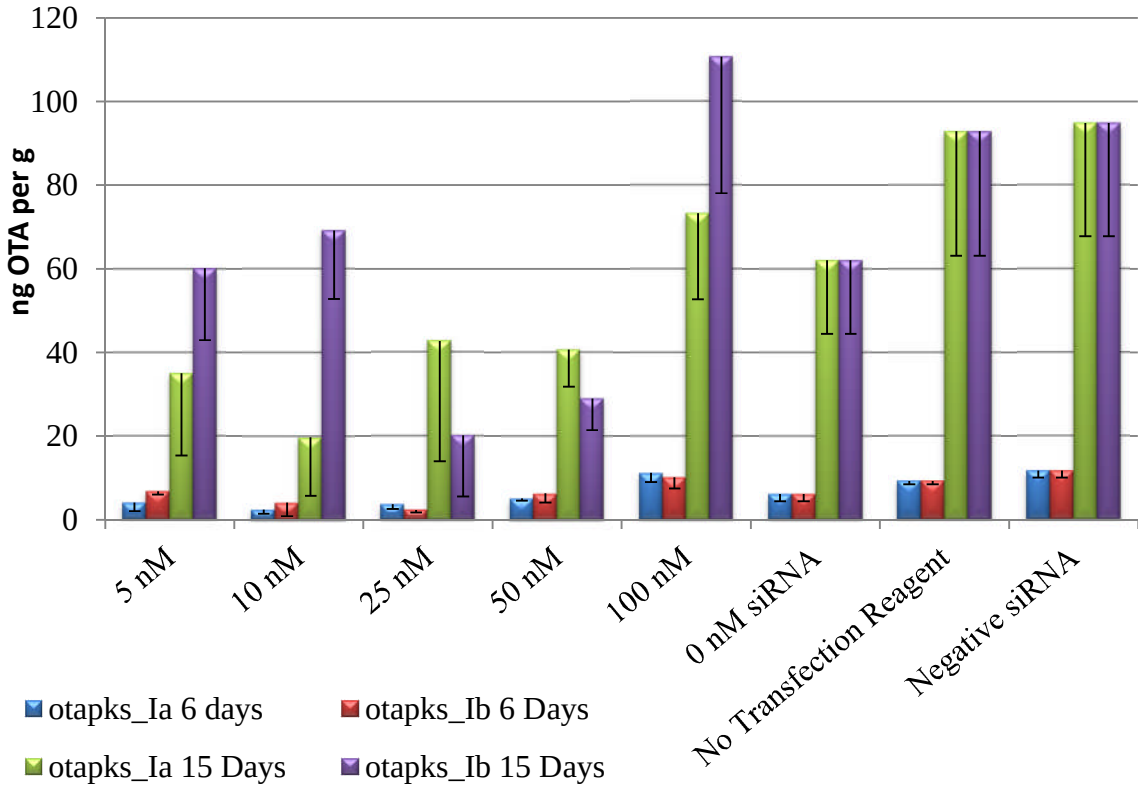

Figure 6.5 - Impact of different siRNA oligonucleotide concentrations on the production of OTA by *P. verrucosum* at 6 and 15 days after transfection. Bars indicate standard error of the mean.

6.3.3 Effect of small interfering RNA on gene expression

The effect of siRNA molecules on the mRNA levels of *otapks*Pv and *Tri5* was assessed after 6 and 15 days using RT-qPCR. *P. verrucosum otapks*Pv gene expression levels in the presence of the siRNA otapks_Ib were consistently lower than expression under the same concentrations of otapks_Ia. Nevertheless compared to the control samples *otapks*Pv gene expression was only reduced when compared to the control with no

siRNA oligonucleotide on day 6 (Figure 6.6) or when compared to *otapks*Pv expression in the presence of a non-related siRNA oligonucleotide after 15 days (Figure 6.7). Moreover there was a detrimental effect of varying the concentration of the siRNA oligonucleotide used.

F. graminearum Tri5 gene expression was down regulated by the presence of both Tri5_Ia and Tri5_Ib 6 days after transfection (Figure 6.8). The effect after 15 days was not as great but there was still some inhibition especially at lower concentrations of the siRNA, Figure 6.9. However, the overall expression of the controls was also reduced if compared to day 6.

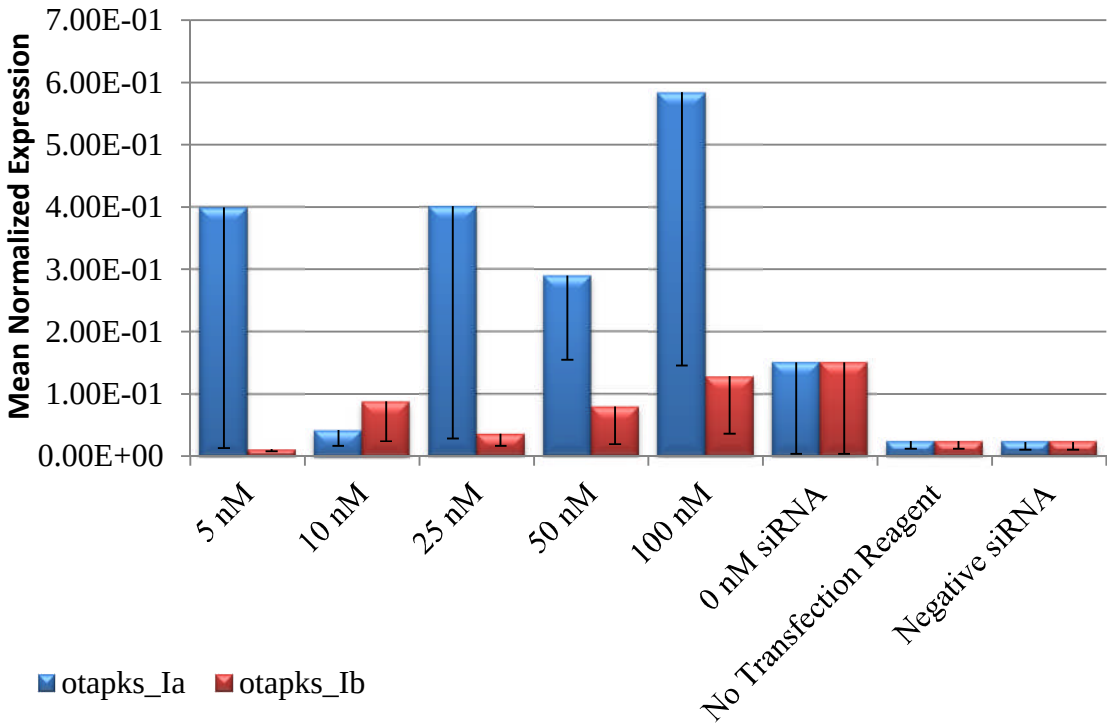

Figure 6.6 - The *otapks*Pv gene expression measured after 6 days after transfection. Vertical bars represent mean standard error.

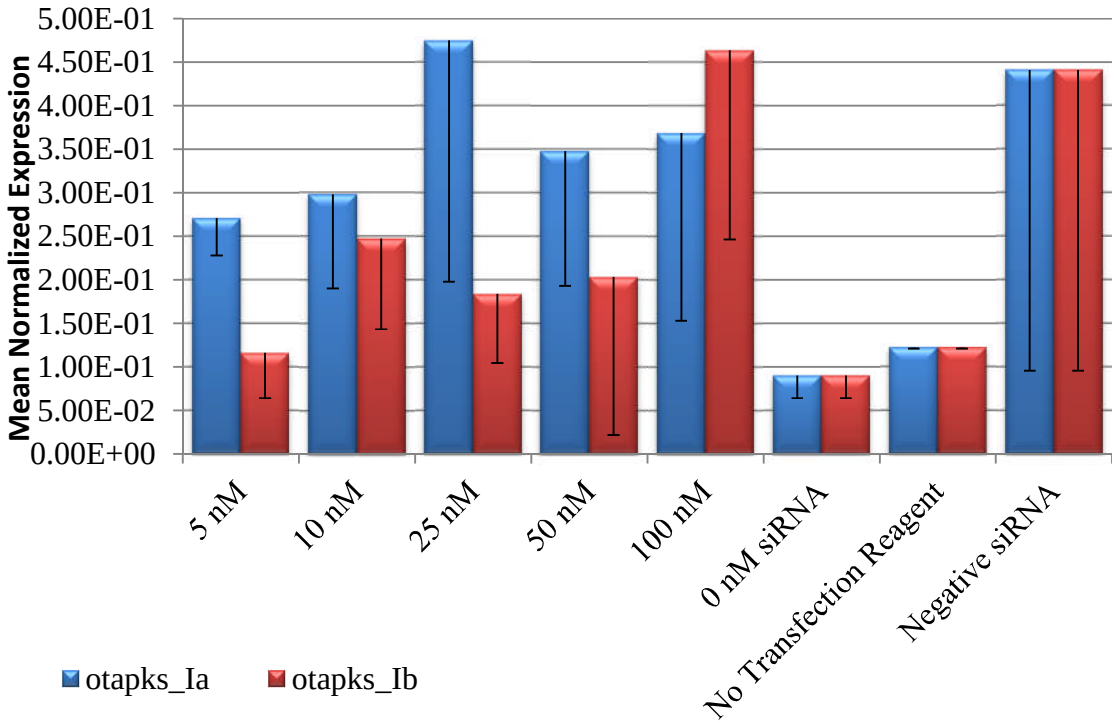

Figure 6.7 - The *otapks*Pv gene expression measured after 12 days after transfection. Vertical bars represent mean standard error.

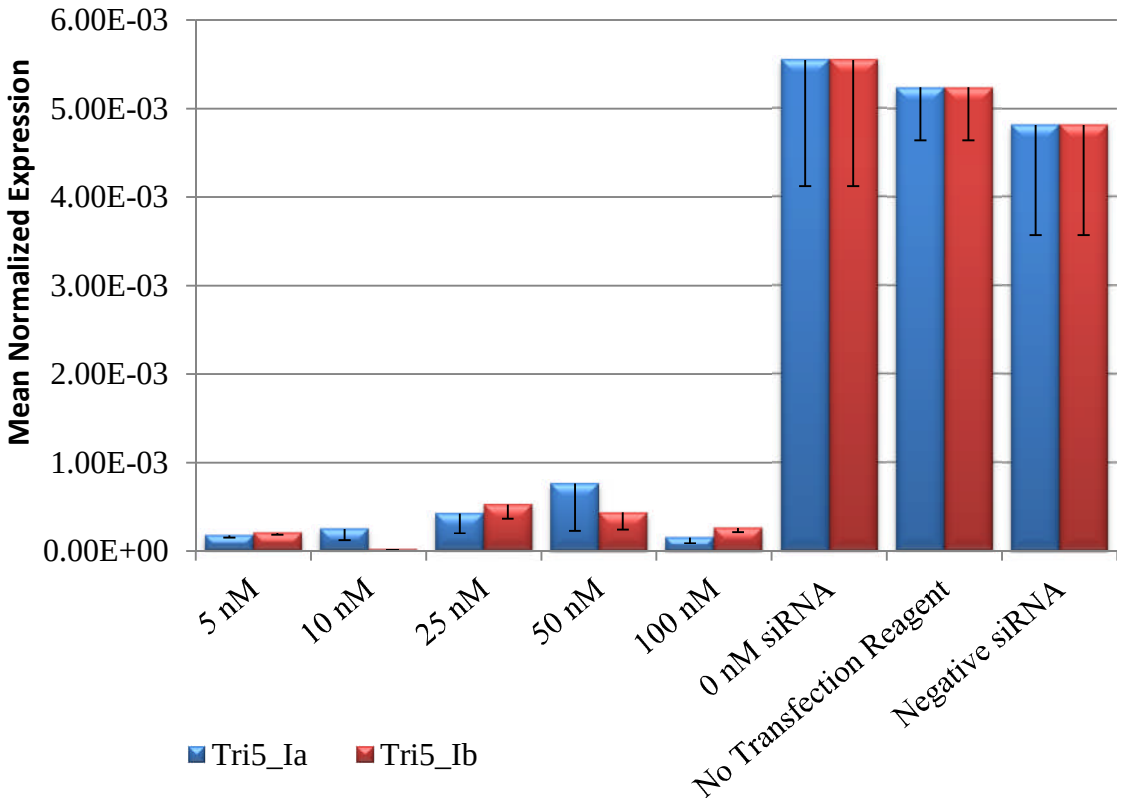

Figure 6.8 - *Tri5* gene expression measured after 6 days after transfection. Vertical bars represent mean standard error.

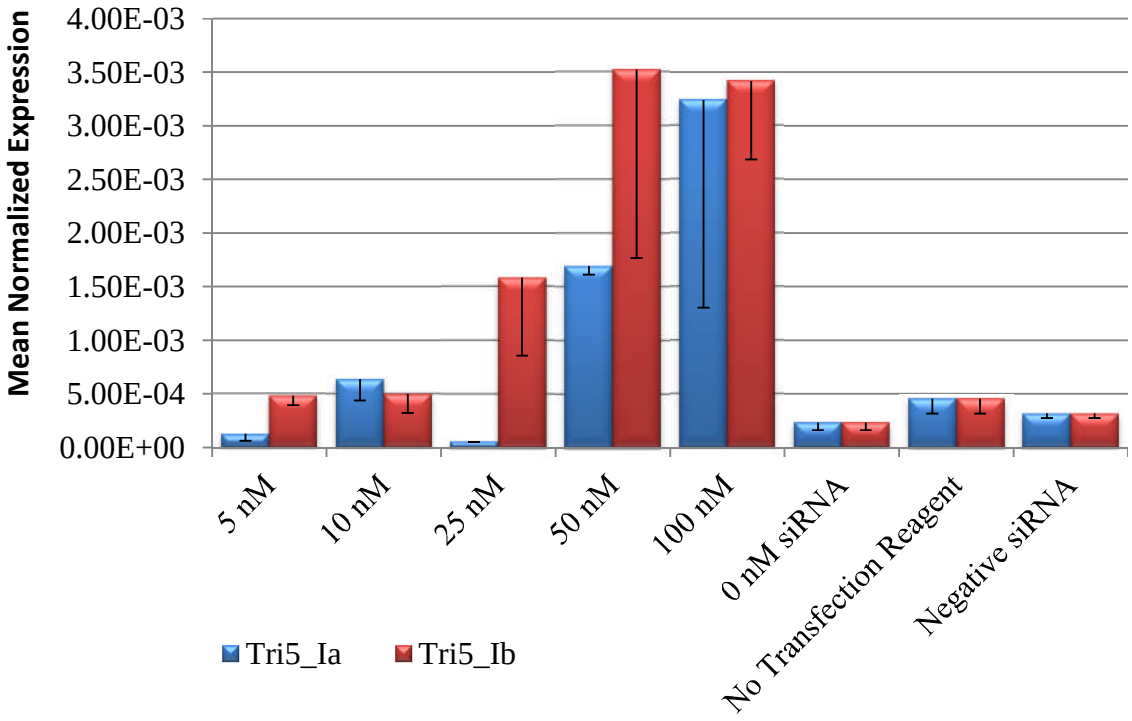

Figure 6.9 - *Tri5* gene expression measured after 15 days after transfection. Vertical bars represent mean standard error.

6.4 Discussion

This is the first study to use RNA interference (RNAi) to silence a key regulatory gene of the ochratoxin A biosynthetic pathway, *otapks*Pv that encodes polyketide synthase. Furthermore, no previous report has shown *P. verrucosum* RNA mediated gene silencing. On the other hand, *F. graminearum Tri6* has been previously reported to have been silenced using RNA interference (McDonald *et al.*, 2005). Nevertheless *Tri5* encodes trichodiene synthase and is also a key regulatory gene in the trichothecene biosynthetic pathway.

The results showed that both siRNA targeted at the *otapks*Pv gene were successful at both toxin and gene expression levels. Even though the designed siRNA had optimum gene silencing at different concentrations, otapks_Ia showed a greater degree of both toxin and gene expression inhibition at 10 nM while otapks_Ib was optimum at 25 nM. These results demonstrate RNA mediated gene silencing in *P. verrucosum* for the first time. The siRNA targeting *Tri5* also showed gene silencing. This however was more evident at day 6, since in the later samples when compared to the control samples only Tri5_Ia reduced *Tri5* gene expression. RNAi gene silencing using siRNA is transient and by day 15th it is possible that gene silencing was no longer being mediated by the transfected siRNA.

Abdel-Hadi *et al.* (2011) found that 25 mM concentration was the most effective for inhibiting an early biosynthetic gene *Afl*D and the regulatory genes *Afl*R/*Afl*S in *Aspergillus flavus* and *A. parasiticus*. It was also suggested that some degree of siRNA uptake from the medium was the reason to observe silenced genes as early as 5 days for *A. flavus and A. parasiticus* (Abdel-Hadi *et al.,* 2011). The results in the present study also suggest gene silencing as soon as 6 days and there was some evidence of direct delivery from the medium in *P. verrucosum* but no such evidence was found for *F. graminearum*. Nevertheless efficient siRNA transfection was only achieved when lipid mediated were around 80% of the protoplast transfected with Cy3 labelled siRNA had increased fluorescence when only 4% of *P. verrucosum* protoplasts with no lipid transfection reagent displayed fluorescence.

Jackson *et al.* (2003) reported silencing of non-targeted genes when using siRNA. The negative control did not lead to gene knockdown and the levels of mycotoxin and gene expression were similar between all controls. Moreover no observable phenotypic change either than the mycotoxin expression was detected with all the siRNA used in this study. The important seed region was complimentary to both genes mRNA and the selected siRNA were chemically modified to reduce off target effects. Nevertheless the increased concentration of siRNA molecules did produce some higher gene expression patterns. These could be the result of off target gene silencing as these have been reported to increase with the concentration of siRNA (Caffrey *et al.*, 2011).

Previously in Chapters 4 and 5 it was shown that key environmental factors and the presence of sub-optimal concentration of antifungal compounds had an impact on the production of mycotoxins. The present study suggests that this could be a useful tool that can be used in a sequence specific manner to inhibit mycotoxin production. Even though the RNAi silencing mechanism has not been subject to different environmental conditions we have shown 3 to 5 times ochratoxin reduction as well as a log reduction on *otapks*Pv and *Tri5*. The use of a stable dsRNA might lead to even higher mycotoxin reduction has reported from *Tri6* silencing (McDonald *et al.*, 2005).

Furthermore, the exogenous expression of dsRNA in the host plant have been previously reported to induce gene silencing in the fungal pathogens *Blumeria graminis* and *Fusarium verticillioides* and also in the parasitic plant *Orobanche aegyptiaca* (Westwood *et al.*, 2009; Nowara *et al.*, 2010; Tinoco *et al.*, 2010). Nevertheless Zhan *et al.* (2011) reported that transgenic expression of dsRNA molecules was not sufficient to initiate gene silencing in *Phytophthora parasitica* during infection of *Arabidopsis thaliana*. Recently Qiao *et al.* (2013) reported that the plant pathogen *Phytophthora sojae* is able to suppress RNA silencing in plants by inhibiting the biogenesis of small RNAs. This can be a specific species virulence factor used during invasion but it can also be the proof of a common gene regulation battle between host defence were RNA silencing appears to have a key role and virulence proteins that target host RNA silencing processes to promote infection. There is a need to further elucidate the exogenous expression of dsRNA by plants and the uptake of

these molecules by their pathogens. The application of RNAi to prevent mycotoxigenic filamentous fungi contamination of commodities needs further studying.

7 Conclusions and future work

7.1 Growth boundaries of spoilage and mycotoxigenic fungi

The initial pH studies allowed the identification of the growth pattern of all 18 strains of spoilage fungi. Furthermore, it showed that the use of a weak acid based buffer also influenced the ability to withstand low pH conditions and reduced the observable growth of several spoilage fungi. In particularly the yeast strains were inhibited at pH 2.5, with the exception of *Zygosaccharomyces bailii*, but more relevant was the effect on *Aspergillus puniceus* and *Penicillium verrucosum*. This was not observed using only HCl as the pH adjusting agent. In that case, only *Saccharomyces cerevisiae* was inhibited at pH 2.5, and even for this strain after 15 days there was some observable growth on one of the replicates. This initial study also showed that the pH would stay stable for the entire study period. Moreover at pH 3.0 all strains tested were capable of growing after 24h, with the exception of one replicate of *S. cerevisiae*, in which growth was only observable after 48h.

The effect of single preservative use to inhibit growth at pH 3.0 showed that sodium benzoate and potassium sorbate were not capable of inhibit visible growth even at concentrations above the EU allowable limit. These findings are of concern for the food industry, since it shows that single attack strategies against spoilage fungi may potentially prove ineffective. Even though this study was not performed as a challenge test, it clearly showed that at pH 3.0 the use of sodium benzoate and potassium sorbate alone was not effective at inhibiting growth. Growth was observable in all tested strains after just 7 days.

The use of multiple preservatives or weak organic acids, on the other hand, showed that growth was effectively inhibited on all strains, except *Aspergillus niger*, within the EU legal limits of use. The most surprisingly result was the capacity that the mixture of sodium benzoate and potassium sorbate had to inhibit all yeasts strains at every concentration combination of both preservatives, and besides *A. niger* no other filamentous fungi was able to grow on the legal limit of this preservatives. Also important was the effect that fumaric and malic acid displayed, and although they

were not as effective at inhibiting growth, mainly the yeast strains grew. They were capable of maintaining inhibition with lower amounts of potassium sorbate, just 200 ppm, in conjunction with these weak organic acids was sufficient to inhibit grow over the entire study period.

7.2 Mycotoxigenic filamentous fungi: key biosynthetic gene and mycotoxin production

Ecophysiological studies of the mycotoxigenic filamentous fungi *F. graminearum* and *P. verrucosum* showed the optimum growth and mycotoxin production conditions for production. In both species the growth was enhanced at high a_W levels even though *P. verrucosum* had a slightly faster growth at 20ºC while *F. graminearum* had the highest growth rate at 25ºC. These are indicative of their natural ecological niches. *P. verrucosum* is a major post-harvest spoilage species in stored commodities in cooler regions, especially northern Europe where it is the main species responsible for OTA contamination. Nevertheless, recent reports have shown its presence in warmer regions including southern Europe. *F. graminearum* is a plant pathogen and important trichothecene producer. Ecophysiological conditions have an important impact on growth rate. *F. graminearum* is globally distributed and has been isolated in all cereal growing regions. Temperature and moisture conditions influence growth rate and may be associated with virulence and FHB disease spread.

Trichothecene production has been associated with grain infection. An overall higher level of *Tri5* expression by *F. graminearum* grown in irradiated grain occurred when compared to YES medium. Moreover water stress conditions led to an increase in *Tri5* expression *in situ* while *in vitro* the expression levels were constant. The presence of sub-lethal and non-optimum concentrations of antifungal compounds not only displayed an overexpression of *Tri5* but also increased amounts of trichothecenes type including DON. Furthermore, in these conditions there was no correspondent increase of *F. graminearum* growth rate. *P. verrucosum* also displayed similar results with sub-optimal concentrations of antifungal compounds leading to OTA stimulation. This was nevertheless more limited while thyme essential oil and Prochloraz at certain water stress conditions lead to an increase of OTA or *otapks*Pv expression. In contrast the

antioxidant BHA was overall more effective. BHA not only affected growth rate but also had the capacity to inhibit *otapks*Pv gene expression and OTA accumulation at all water stress conditions tested.

7.3 RNAi approaches to control deoxynivalenol and ochratoxin A production by *F. graminearum* and *P. verrucosum*

RNAi is a novel control strategy for reducing mycotoxin contamination the results showed that at least at one of the concentrations tested, the siRNA were effective at reducing toxin and mycotoxigenic gene expression. siRNA molecules induce transient RNA mediated gene silencing and as such the observed levels of *otapks*Pv expression after 15 days post transfection represented the loss of gene silencing. Nevertheless, OTA levels were still reduced. This is the first study to show RNA mediated gene silencing in *P. verrucosum* which also confirms that this spoilage species has the required molecular mechanisms for RNAi. This can facilitate the needed genomic studies into the elucidation of key gene functions in this mycotoxigenic fungus.

Even though *F. graminearum* has previously been subject to RNAi this was the first study to present *Tri5* gene knockdown and unlike *otapks*Pv gene silencing, *Tri5* silencing was still observable after 15 days post transfection with Tri5_Ia. Trichothecenes type B were below the LOD and so the effect on them could not be assessed. Nevertheless, the level of gene silencing and previous knockout experiments of this gene indicate that they also may be inhibited.

The concentration of the siRNA oligonucleotide can have a profound influence on their efficacy with the higher concentrations promoting not only gene but also mycotoxin production as was observed with 100 nM of otapks_Ib. However, this concentration was 5 times above the optimum which for this siRNA was 25 nM. In fact, all siRNA used had optimum concentrations of between 5 and 25 nM.

7.4 New control strategies trough development of new methods

A rapid high throughput RNA extraction method has been developed. Overall 3 times higher yields of total RNA were achieved using this method. Moreover the integrity

and quality of the RNA was enhanced by using the method developed. Also the reduction of time and cross contamination risk represent an improvement that allied with the lower starting material requirements can be advantageous for future high throughput screening studies. The bead beating protocol developed allowed the extraction of high quality RNA used throughout this project.

While preservatives and antifungal compounds commonly used at different stages of the food chain have presented varying degrees of success. There was no single preservative or antifungal compound capable of inhibiting both growth and mycotoxin production at all conditions and spoilage fungi. This study has challenged commonly used spoilage fungi control strategies at different key points of the food chain, and has shown their efficacy while presenting alternatives to consumer and new regulatory pressures. At all stages from seed selection, harvest, post-harvest till food industry production we have presented the risks involved in facilitating fungal spoilage.

The alternative control strategy presented by the chance of inhibiting on a sequence specific manner key biosynthetic pathway genes is a possibility to the mycotoxigenic species of *F. graminearum* and *P. verrucosum*. This approach can lead to more accurate mycotoxin control strategies either by targeting specif genes or attempting to target key enzymes. The result can be the development of a method to control mycotoxin contamination with reduced ecological interference.

7.5 Suggestions for future work.

- Assessment of malic and fumaric mixtures to control spoilage fungi in low nutrient conditions.
- Develop growth boundaries predictive model.
- Investigation of the effect of mixtures of antifungal compounds over mycotoxigenic filamentous fungi grown on wheat.
- Temporal studies of key mycotoxigenic genes at different biotic and abiotic conditions.
- Assess the possibility to use enzyme blockers to inhibit tricodiene synthethase and OTA polyketide synthase activity.

- Research the possibility to use inverted repeat transgenes (IRT) containing homologues sequences to *Tri5* and *otapks*Pv gene to regulate these genes by RNA silencing.
- Investigate the prospect to use plant production of dsRNA molecules with sequences corresponding to *Tri5* and *otapks*Pv gene to regulate these genes during infection.

Publications

Peer reviewed publications

- Leite, G. M., Magan, N. and Medina, A. (2012) "Comparison of different bead-beating RNA extraction strategies: an optimized method for filamentous fungi" *Journal of Microbiological Methods,* 88, 413-418.
- Medina A., Leite G. M. and Magan N. "Simultaneous HPLC-DAD analysis of Nivalenol, Deoxynivalenol, 15- and 3-Acetyldeoxynivalenol from wheat using a new solid core particle C_{18} column". Manuscript under review.

Oral Presentations

- The impact of anti-fungal compounds and environmental stress interactions on *Fusarium graminearum* growth and *TRI5* gene expression. In International Society of Mycotoxicology 2011 conference, Mycored 2011, Strategies to reduce the impact of mycotoxins in Latin America in a global context, Mendonza, Argentina, 15-18 November 2011.
- Effects of anti-fungal compounds on *Fusarium graminearum* growth and *Tri5* gene expression. In Cranfield Health Postgraduate Student Conference, Cranfield, UK, 23th of September 2011

Poster Presentations

- The impact of anti-fungal compounds and environmental stress interactions on *Fusarium graminearum* growth and *Tri5* gene expression. Gonçalo Leite and Naresh Magan. British Mycological Society 2011, Fungal Development ad Pathogenesis, Exeter, UK, 13-16 September 2011.
- The impact of interactions between anti-fungal compounds and environmental stress on growth of *Fusarium graminearum* and deoxynivalenol production using the *Tri5* gene. Gonçalo Leite and Naresh Magan. Unilever Symposium, Colworth, Bedford, UK, 23rd of June 2011.
- Optimization of the total RNA extraction from mycotoxigenic fungi using a bead beating protocol. Gonçalo Leite, Naresh Magan and Angel Medina. International Mycotoxin Conference, MycoRed, 2010, Global Mycotoxin

Reduction Strategies: Asia and Pacific Rim, Penang, Malaysia, 1-4 December 2010.

- Study of the growth boundaries of 17 spoilage fungi of beverages. Gonçalo Leite, Ronald J. W. Lambert and Naresh Magan. Cranfield Health Postgraduate Student Conference, Cranfield, UK, 22nd of September 2010.

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Appendix A

A.1 Effect of pH and pH buffering system on the growth boundary of spoilage fungi.

Spoilage Yeast Strains	pH	Visual examination scheme (days)																															
		1				2				3				4				5				7				10				15				20			
		S1	S2	S3	MC	S1	S2	S3	MC	S1	S2	S3	MC	S1	S2	S3	MC	S1	S2	S3	MC	S1	S2	S3	MC	S1	S2	S3	MC	S1	S2	S3	MC	S1	S2	S3	MC
Y10 - *Candida albicans*	2,5																																		
	3.0									+*				+*				+*				+*				+*				+*				+*			
	3,5	■	■	■		■	■	■		■	■	■		■	■	■		■	■	■		■	■	■		■	■	■		■	■	■		■	■	■	
	4.0	■	■	■		■	■	■		■	■	■		■	■	■		■	■	■		■	■	■		■	■	■		■	■	■		■	■	■	
Y2 - *Candida parapsilosis*	2,5																																		
	3.0						■	■		■	■	■		■	■	■		■	■	■		■	■	■		■	■	■		■	■	■		■	■	■	
	3,5	■	■	■		■	■	■		■	■	■		■	■	■		■	■	■		■	■	■		■	■	■		■	■	■		■	■	■	
	4.0	■	■	■		■	■	■		■	■	■		■	■	■		■	■	■		■	■	■		■	■	■		■	■	■		■	■	■	
Y9 - *Candida tropicalis*	2,5																																		
	3.0																														+*				+*		
	3,5	■	■	■		■	■	■		■	■	■		■	■	■		■	■	■		■	■	■		■	■	■		■	■	■		■	■	■	
	4.0	■	■	■		■	■	■		■	■	■		■	■	■		■	■	■		■	■	■		■	■	■		■	■	■		■	■	■	
Y13 - *Clavispora lusitaniae*	2,5																																		
	3.0							■		■	■	■		■	■	■		■	■	■		■	■	■		■	■	■		■	■	■		■	■	■	
	3,5	■	■	■		■	■	■		■	■	■		■	■	■		■	■	■		■	■	■		■	■	■		■	■	■		■	■	■	
	4.0	■	■	■		■	■	■		■	■	■		■	■	■		■	■	■		■	■	■		■	■	■		■	■	■		■	■	■	
Y12 - *Pichia menbranafaciens*	2,5																																		
	3.0					■	■	■		■	■	■		■	■	■		■	■	■		■	■	■		■	■	■		■	■	■		■	■	■	
	3,5	■	■	■		■	■	■		■	■	■		■	■	■		■	■	■		■	■	■		■	■	■		■	■	■		■	■	■	
	4.0	■	■	■		■	■	■		■	■	■		■	■	■		■	■	■		■	■	■		■	■	■		■	■	■		■	■	■	
Y11 - *Saccharomyces cerevisiae*	2,5																																		
	3.0	■	■	■		■	■	■		■	■	■		■	■	■		■	■	■		■	■	■		■	■	■		■	■	■		■	■	■	
	3,5	■	■	■		■	■	■		■	■	■		■	■	■		■	■	■		■	■	■		■	■	■		■	■	■		■	■	■	
	4.0	■	■	■		■	■	■		■	■	■		■	■	■		■	■	■		■	■	■		■	■	■		■	■	■		■	■	■	
Y1 - *Zygosaccharomyces bailli*	2,5	■				■	■	■		■	■	■		■	■	■		■	■	■		■	■	■		■	■	■		■	■	■		■	■	■	
	3.0	■	■	■		■	■	■		■	■	■		■	■	■		■	■	■		■	■	■		■	■	■		■	■	■		■	■	■	
	3,5	■	■	■		■	■	■		■	■	■		■	■	■		■	■	■		■	■	■		■	■	■		■	■	■		■	■	■	
	4.0	■	■	■		■	■	■		■	■	■		■	■	■		■	■	■		■	■	■		■	■	■		■	■	■		■	■	■	

Figure A.1 - Detailed observations over 20 days, of yeasts cultures at different pH media, adjusted using KH-Phthalate buffer. S1, S2 and S3 represent each strain replicate and MC represents the media sterility control. ■ - Observable Growth, □ – No Observable Growth, +*- Reduced observable growth

Filamentous fungi Strains	pH	Visual examination scheme (days)																													
		1				2				3				4				5				7				10				15				20			
		S1	S2	S3	MC	S1	S2	S3	MC	S1	S2	S3	MC	S1	S2	S3	MC	S1	S2	S3	MC	S1	S2	S3	MC	S1	S2	S3	MC	S1	S2	S3	MC	S1	S2	S3	MC
Y20 - *Aspergillus flavus**	2,5	■	■	■	□	■	■	■	□	■	■	■	□				□				□				□			S	□	S	S	S	□	S	S	S	□
	3.0	■	■	■	□	■	■	■	□	■	■	■	□			S	□		S	S	□		S	S	□	S	S	S	□	S	S	S	□	S	S	S	□
	3,5	■	■	■	□	■	■	■	□	■	■	■	□	S	S	S	□	S	S	S	□	S	S	S	□	S	S	S	□	S	S	S	□	S	S	S	□
	4.0	■	■	■	□	■	■	■	□	■	■	■	□	S	S	S	□	S	S	S	□	S	S	S	□	S	S	S	□	S	S	S	□	S	S	S	□
Y14 - *Aspergillus niger*	2,5	■	■	■	□	■	■	■	□	■	■	■	□				□				□				□			S	□	S	S	S	□	S	S	S	□
	3.0	■	■	■	□	■	■	■	□	■	■	■	□	S	S	S	□	S	S	S	□	S	S	S	□	S	S	S	□	S	S	S	□	S	S	S	□
	3,5	■	■	■	□	■	■	■	□	■	■	■	□		S		□	S	S	S	□	S	S	S	□	S	S	S	□	S	S	S	□	S	S	S	□
	4.0	■	■	■	□	■	■	■	□	■	■	■	□	S			□	S			□	S	S		□	S	S		□	S	S	S	□	S	S	S	□
Y15 - *Aspergillus puniceus*	2,5	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□
	3.0	□	□	□	□	■	□	□	□	■	□	□	□	■	□	□	□	■	■	□	□	■	■	□	□	■	□	□	□	■	■	□	□	■	■	□	□
	3,5	□	□	□	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□
	4.0	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□
Y19 - *Fusarium graminearum**	2,5	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□
	3.0	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□
	3,5	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□
	4.0	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□
Y3 - *Fusarium oxysporum*	2,5	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	S	S	S	□
	3.0	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	S	■	□	■	S	■	□	■	S	■	□	S	S	S	□
	3,5	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	S	S	■	□	S	S	■	□	S	S	■	□	S	S	■	□
	4.0	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	S	■	□	■	S	■	□	■	S	S	□	S	S	S	□
Y6 - *Penicillium biourgeianum*	2,5	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	S	S	□	■	S	S	□	■	S	S	□
	3.0	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	S	S	□	■	S	S	□	■	S	S	□
	3,5	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	S	S	■	□	S	S	■	□	S	S	■	□
	4.0	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	S	S	S	□	S	S	S	□	S	S	S	□
Y5 - *Penicillium citreonigrum*	2,5	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	S	□	■	■	S	□	S	■	S	□	S	■	S	□
	3.0	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	S	■	■	□	S	■	■	□
	3,5	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	S	■	□	■	S	■	□	■	S	■	□	S	S	■	□
	4.0	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□
Y18 - *Penicillium corylophylum*	2,5	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	S	S	S	□
	3.0	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	S	S	S	□	S	S	S	□
	3,5	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	S	S	□	S	S	S	□	S	S	S	□	S	S	S	□
	4.0	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	S	S	S	□	S	S	S	□	S	S	S	□	S	S	S	□
Y8 - *Penicillium echinulatum*	2,5	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	S	□
	3.0	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	S	S	S	□	S	S	S	□	S	S	S	□	S	S	S	□
	3,5	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	S	S	S	□	S	S	S	□	S	S	S	□	S	S	S	□
	4.0	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	S	S	S	□	S	S	S	□	S	S	S	□	S	S	S	□
Y4 - *Penicillium glabrum*	2,5	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	S	□	■	■	S	□	■	■	S	□
	3.0	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	S	S	□	■	S	S	□	■	S	S	□	■	S	S	□
	3,5	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	S	S	S	□	S	S	S	□
	4.0	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	S	S	S	□	S	S	S	□
Y16 - *Penicillium glabrum*	2,5	■	□	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□
	3.0	■	□	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	S	■	■	□	S	■	■	□	S	■	■	□	S	■	■	□
	3,5	■	□	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	S	■	□	■	S	S	□	S	S	S	□	S	S	S	□
	4.0	■	□	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	S	S	□	■	S	S	□	S	S	S	□	S	S	S	□
Y17 - *Penicillium spinolosum*	2,5	■	□	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	S	S	□	S	S	S	□	S	S	S	□
	3.0	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	S	S	S	□	S	S	S	□	S	S	S	□
	3,5	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	S	S	□	S	S	S	□	S	S	S	□	S	S	S	□
	4.0	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	S	S	S	□	S	S	S	□	S	S	S	□	S	S	S	□
Y7 - *Penicillium verucosum**	2,5	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□
	3.0	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□
	3,5	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□
	4.0	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□

Figure A.2 - Detailed observations over 20 days, of spoilage filamentous fungi cultures at different pH media, adjusted using KH-Phthalate buffer. S1, S2 and S3 represent each strain replicate and MC represents the media sterility control. ■ - Observable Growth, □ – No Observable Growth, S- Observable Conidia Formation *-Mycotoxigenic Strain

Yeast Strains	pH	Visual examination scheme (days)																													
		1				2				3				4				5				7				10				15			
		S1	S2	S3	MC	S1	S2	S3	MC	S1	S2	S3	MC	S1	S2	S3	MC	S1	S2	S3	MC	S1	S2	S3	MC	S1	S2	S3	MC	S1	S2	S3	MC
Y10 - *Candida albicans*	2,5	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□
	3.0	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□
	3,5	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□
	4.0	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□
Y2 - *Candida parapsilosis*	2,5	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□
	3.0	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□
	3,5	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□
	4.0	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□
Y9 - *Candida tropicalis*	2,5	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□
	3.0	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□
	3,5	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□
	4.0	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□
Y13 - *Clavispora lusitaniae*	2,5	□	□	□	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□
	3.0	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□
	3,5	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□
	4.0	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□
Y12 - *Pichia menbranafaciens*	2,5	□	□	□	□	□	□	□	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□
	3.0	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□
	3,5	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□
	4.0	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□
Y11 - *Saccharomyces cerevisiae*	2,5	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	+*	□	□
	3.0	□	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□
	3,5	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□
	4.0	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□
Y1 - *Zygosaccharomyces bailli*	2,5	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□
	3.0	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□
	3,5	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□
	4.0	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□

Figure A.3 - Detailed observations over 15 days, of yeast cultures at different pH media, adjusted using HCl. S1, S2 and S3 represent each strain replicate and MC represents the media sterility control. ■ - Observable Growth, □ – No Observable Growth, +*- Reduced observable growth

Filamentous fungi Strains	pH	Visual examination scheme (days)																													
		1				2				3				4				5				7				10				15			
		S1	S2	S3	MC	S1	S2	S3	MC	S1	S2	S3	MC	S1	S2	S3	MC	S1	S2	S3	MC	S1	S2	S3	MC	S1	S2	S3	MC	S1	S2	S3	MC
Y20 - *Aspergillus flavus**	2,5	■	■	■	□	■	■	■	□	□	□	□	□	□	□	□	□	□	□	□	□	S	□	□	□	S	S	S	□	S	S	S	□
	3.0	■	■	■	□	■	■	■	□	□	S	S	□	□	S	S	□	S	S	S	□	S	S	S	□	S	S	S	□	S	S	S	□
	3,5	■	■	■	□	■	■	■	□	S	S	S	□	S	S	S	□	S	S	S	□	S	S	S	□	S	S	S	□	S	S	S	□
	4.0	■	■	■	□	■	■	■	□	S	S	S	□	S	S	S	□	S	S	S	□	S	S	S	□	S	S	S	□	S	S	S	□
Y14 - *Aspergillus niger*	2,5	■	■	■	□	■	■	■	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	S	S	S	□	S	S	S	□
	3.0	■	■	■	□	■	■	■	□	□	□	□	□	□	S	S	□	S	S	S	□	S	S	S	□	S	S	S	□	S	S	S	□
	3,5	■	■	■	□	■	■	■	□	□	□	□	□	S	S	S	□	S	S	S	□	S	S	S	□	S	S	S	□	S	S	S	□
	4.0	■	■	■	□	■	■	■	□	□	□	□	□	S	S	S	□	S	S	S	□	S	S	S	□	S	S	S	□	S	S	S	□
Y15 - *Aspergillus puniceus*	2,5	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□
	3.0	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□
	3,5	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□
	4.0	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□
Y19 - *Fusarium graminearum**	2,5	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□
	3.0	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□
	3,5	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□
	4.0	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□
Y3 - *Fusarium oxysporum*	2,5	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	S	S	S	□	S	S	S	□
	3.0	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	S	S	S	□	S	S	S	□
	3,5	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	S	S	S	□	S	S	S	□
	4.0	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	S	■	□	S	S	S	□	S	S	S	□
Y6 - *Penicillium biourgeianum*	2,5	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	S	■	S	□	S	S	S	□
	3.0	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	S	■	S	□	S	S	S	□	S	S	S	□
	3,5	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	S	S	S	□	S	S	S	□	S	S	S	□
	4.0	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	S	S	S	□	S	S	S	□	S	S	S	□
Y5 - *Penicillium citreonigrum*	2,5	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	S	S	S	□	S	S	S	□	S	S	S	□
	3.0	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	S	■	■	□	S	S	S	□	S	S	S	□
	3,5	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	S	S	S	□	S	S	S	□	S	S	S	□
	4.0	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	S	S	S	□	S	S	S	□	S	S	S	□
Y18 - *Penicillium corylophylum*	2,5	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	S	S	□	■	S	S	□	■	S	S	□
	3.0	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	S	S	■	□	S	S	S	□	S	S	S	□
	3,5	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	S	S	□	■	S	S	□	■	S	S	□
	4.0	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	S	■	□	■	S	■	□	■	S	■	□
Y8 - *Penicillium echinulatum*	2,5	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	S	S	S	□	S	S	S	□
	3.0	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	S	S	S	□	S	S	S	□	S	S	S	□
	3,5	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	S	S	S	□	S	S	S	□	S	S	S	□
	4.0	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	S	S	S	□	S	S	S	□	S	S	S	□
Y4 - *Penicillium glabrum*	2,5	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	S	S	S	□	S	S	S	□
	3.0	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	S	■	■	□	S	S	S	□	S	S	S	□
	3,5	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	S	■	■	□	S	S	S	□	S	S	S	□
	4.0	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	S	S	S	□	S	S	S	□
Y16 - *Penicillium glabrum*	2,5	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□
	3.0	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□
	3,5	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	S	■	■	□	S	■	■	□	S	■	■	□
	4.0	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	S	■	□	■	S	■	□	■	S	■	□
Y17 - *Penicillium spinolosum*	2,5	■	■	■	□	■	■	■	□	■	■	■	□	■	S	■	□	■	S	■	□	■	S	■	□	S	S	S	□	S	S	S	□
	3.0	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	S	□	S	■	S	□	S	S	S	□	S	S	S	□
	3,5	■	■	■	□	■	■	■	□	■	■	■	□	S	■	S	□	S	■	S	□	S	■	S	□	S	■	S	□	S	■	S	□
	4.0	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	S	■	□	■	■	■	□	■	■	■	□
Y7 - *Penicillium verucosum**	2,5	□	□	□	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□
	3.0	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□
	3,5	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□
	4.0	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□

Figure A.4 - Detailed observations over 15 days, of spoilage yeast cultures at different pH media, adjusted using HCl. S1, S2 and S3 represent each strain replicate and MC represents the media sterility control. ■ - Observable Growth, □ – No Observable Growth, S- Observable Conidia Formation, *- Mycotoxigenic Strain

A.2 Effect of single preservative/weak organic acid solutions on the growth boundary of spoilage fungi.

Yeast Strains	|B| (PPM)	Visual examination scheme (days)																													
		1				2				3				4				5				7				10				15			
		S1	S2	S3	MC	S1	S2	S3	MC	S1	S2	S3	MC	S1	S2	S3	MC	S1	S2	S3	MC	S1	S2	S3	MC	S1	S2	S3	MC	S1	S2	S3	MC
Y10 - *Candida albicans*	0	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□
	50	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□
	100	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□
	150	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□
	200	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□
Y2 - *Candida parapsilosis*	0	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□
	50	□	□	□	□	□	□	□	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□
	100	□	□	□	□	□	□	□	□	□	□	□	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□
	150	□	□	□	□	□	□	□	□	□	□	□	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□
	200	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□
Y9 - *Candida tropicalis*	0	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□
	50	□	□	□	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□
	100	□	□	□	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□
	150	□	□	□	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□
	200	□	□	□	□	□	□	□	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□
Y13 - *Clavispora lusitaniae*	0	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□
	50	□	□	□	□	□	□	□	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□
	100	□	□	□	□	□	□	□	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□
	150	□	□	□	□	□	□	□	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□
	200	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□
Y12 - *Pichia menbranafaciens*	0	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□
	50	□	□	□	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□
	100	□	□	□	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□
	150	□	□	□	□	□	■	□	□	■	■	□	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□
	200	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□
Y11 - *Saccharomyces cerevisiae*	0	□	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□
	50	□	□	□	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□
	100	□	□	□	□	□	■	□	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□
	150	□	□	□	□	□	□	□	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□
	200	□	□	□	□	□	□	□	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□
Y1 - *Zygosaccharomyces bailli*	0	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□
	50	□	□	□	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□
	100	□	□	□	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□
	150	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□
	200	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□

Figure A.5 - Detailed observations over 15 days, of the effect of sodium benzoate on yeast strains. S1, S2 and S3 represent each strain replicate and MC represents the media sterility control. ■ - Observable Growth, □ – No Observable Growth

Filamentous fungi Strains	|B| (PPM)	Visual examination scheme (days)																													
		1				2				3				4				5				7				10				15			
		S1	S2	S3	MC	S1	S2	S3	MC	S1	S2	S3	MC	S1	S2	S3	MC	S1	S2	S3	MC	S1	S2	S3	MC	S1	S2	S3	MC	S1	S2	S3	MC
Y20 - *Aspergillus flavus**	0	■	■	■	□	■	■	■	□	■	S	S	□	■	S	S	□	■	■	■	□	S	S	S	□	S	S	S	□	S	S	S	□
	50	□	□	□	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	S	S	S	□	S	S	S	□	S	S	S	□
	100	□	□	□	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	S	S	□	S	S	S	□	S	S	S	□
	150	□	□	□	□	□	□	□	□	□	□	□	□	■	□	□	□	■	■	■	□	■	■	■	□	■	■	■	□	S	S	S	□
	200	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□
Y14 - *Aspergillus niger*	0	■	■	■	□	■	■	■	□	■	■	■	□	■	S	S	□	■	S	S	□	■	S	S	□	S	S	S	□	S	S	S	□
	50	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□
	100	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□
	150	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□
	200	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□
Y15 - *Aspergillus puniceus*	0	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□
	50	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□
	100	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□
	150	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□
	200	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□
Y19 - *Fusarium graminearum**	0	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□
	50	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□
	100	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□
	150	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□
	200	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□
Y3 - *Fusarium oxysporum*	0	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	S	S	S	□
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	200	□	□	□	□	□	□	□	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□
Y6 - *Penicillium biourgeianum*	0	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	S	■	S	□	S	S	S	□	S	S	S	□
	50	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□
	100	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□
	150	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□
	200	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□
Y5 - *Penicillium citreonigrum*	0	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	S	■	■	□	S	S	■	□	S	S	S	□
	50	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□
	100	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□
	150	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□
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Figure A.6 - Detailed observations over 15 days, of the effect of sodium benzoate on filamentous fungi. S1, S2 and S3 represent each strain replicate and MC represents the media sterility control. ■ - Observable Growth, □ – No Observable Growth, S- Observable Conidia Formation *- Mycotoxigenic Strain

Filamentous fungi Strains (Cont.)	|B| (PPM)	Visual examination scheme (days)																													
		1				2				3				4				5				7				10				15			
		S1	S2	S3	MC	S1	S2	S3	MC	S1	S2	S3	MC	S1	S2	S3	MC	S1	S2	S3	MC	S1	S2	S3	MC	S1	S2	S3	MC	S1	S2	S3	MC
Y18 - *Penicillium corylophylum*	0	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	S	S	S	□	S	S	S	□	S	S	S	□
	50	□	□	□	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□
	100	□	□	□	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□
	150	□	□	□	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□
	200	□	□	□	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□
Y8 - *Penicillium echinulatum*	0	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	S	S	S	□	S	S	S	□	S	S	S	□
	50	□	□	□	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□
	100	□	□	□	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□
	150	□	□	□	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□
	200	□	□	□	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□
Y4 - *Penicillium glabrum*	0	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	S	■	■	□	S	S	■	□	S	S	S	□
	50	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□
	100	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□
	150	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□
	200	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□
Y16 - *Penicillium glabrum*	0	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□
	50	□	□	□	□	□	□	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□
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	150	□	□	□	□	□	□	□	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□
	200	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□
Y17 - *Penicillium spinolosum*	0	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	S	■	S	□	S	■	S	□	S	■	S	□
	50	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□
	100	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□
	150	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□
	200	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□
Y7 - *Penicillium verucosum**	0	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□
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	150	□	□	□	□	□	□	□	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□
	200	□	□	□	□	□	□	□	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□

Figure A.7 - Detailed observations over 15 days, of the effect of sodium benzoate on filamentous fungi. S1, S2 and S3 represent each strain replicate and MC represents the media sterility control. ■ - Observable Growth, □ – No Observable Growth, S- Observable Conidia Formation *- Mycotoxigenic Strain

Yeast Strains	[S] (PPM)	Visual examination scheme (days)																													
		1				2				3				4				5				7				10				15			
		S1	S2	S3	MC	S1	S2	S3	MC	S1	S2	S3	MC	S1	S2	S3	MC	S1	S2	S3	MC	S1	S2	S3	MC	S1	S2	S3	MC	S1	S2	S3	MC
Y10 - *Candida albicans*	0	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□
	100	□	□	□	□	□	□	□	□	■	■	■	□	□	□	□	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□
	200	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□
	300	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□
	400	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	■	■	■	□	■	■	■	□	■	■	■	□
Y2 - *Candida parapsilosis*	0	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□
	100	□	□	□	□	□	□	□	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□
	200	□	□	□	□	□	□	□	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□
	300	□	□	□	□	□	□	□	□	□	□	□	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□
	400	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□
Y9 - *Candida tropicalis*	0	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□
	100	□	□	□	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□
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Y13 - *Clavispora lusitaniae*	0	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□
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	400	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	■	■	■	□	■	■	■	□	■	■	■	□
Y12 - *Pichia menbranafaciens*	0	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□
	100	□	□	□	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□
	200	□	□	□	□	□	□	□	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□
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Y11 - *Saccharomyces cerevisiae*	0	□	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□
	100	□	□	□	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□
	200	□	□	□	□	□	□	□	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□
	300	□	□	□	□	□	□	□	□	□	□	□	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□
	400	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□
Y1 - *Zygosaccharomyces bailli*	0	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□
	100	□	□	□	□	□	□	□	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□
	200	□	□	□	□	□	□	□	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□
	300	□	□	□	□	□	□	□	□	□	□	□	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□
	400	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□

Figure A.8 - Detailed observations over 15 days, of the effect of potassium sorbate on yeast strains. S1, S2 and S3 represent each strain replicate and MC represents the media sterility control. ■ - Observable Growth, □ – No Observable Growth

Filamentous fungi Strains	|S| (PPM)	Visual examination scheme (days)																													
		1				2				3				4				5				7				10				15			
		S1	S2	S3	MC	S1	S2	S3	MC	S1	S2	S3	MC	S1	S2	S3	MC	S1	S2	S3	MC	S1	S2	S3	MC	S1	S2	S3	MC	S1	S2	S3	MC
Y20 - *Aspergillus flavus**	0	■	■	■	□	■	■	■	□	■	S	S	□	■	S	S	□	S	S	S	□	S	S	S	□	S	S	S	□	S	S	S	□
	100	□	□	□	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	S	S	S	□
	200	□	□	□	□	□	□	□	□	□	□	□	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□
	300	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	■	■	■	□
	400	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□
Y14 - *Aspergillus niger*	0	■	■	■	□	■	■	■	□	■	■	■	□	■	S	S	□	S	S	S	□	S	S	S	□	S	S	S	□	S	S	S	□
	100	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□
	200	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□
	300	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□
	400	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□
Y15 - *Aspergillus puniceus*	0	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□
	100	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□
	200	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□
	300	□	□	□	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□
	400	□	□	□	□	□	□	□	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□
Y19 - *Fusarium graminearum**	0	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□
	100	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□
	200	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□
	300	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□
	400	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□
Y3 - *Fusarium oxysporum*	0	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	S	S	S	□
	100	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□
	200	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□
	300	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□
	400	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□
Y6 - *Penicillium biourgeianum*	0	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	S	S	S	□	S	S	S	□	S	S	S	□
	100	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□
	200	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□
	300	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□
	400	□	□	□	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□

Figure A.9 - Detailed observations over 15 days, of the effect of potassium sorbate on filamentous fungi. S1, S2 and S3 represent each strain replicate and MC represents the media sterility control. ■ - Observable Growth, □ – No Observable Growth, S- Observable Conidia Formation *- Mycotoxigenic Strain

Filamentous fungi Strains (cont.)	|S| (PPM)	Visual examination scheme (days)																													
		1				2				3				4				5				7				10				15			
		S1	S2	S3	MC	S1	S2	S3	MC	S1	S2	S3	MC	S1	S2	S3	MC	S1	S2	S3	MC	S1	S2	S3	MC	S1	S2	S3	MC	S1	S2	S3	MC
Y5 - *Penicillium citreonigrum*	0	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	S			□	S	S		□	S	S	S	□
	100	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□				□				□				□
	200	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□				□				□				□
	300	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□				□				□				□
	400	□	□	□	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□				□				□				□
Y18 - *Penicillium corylophylum*	0	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	S	S	S	□	S	S	S	□	S	S	S	□
	100	□	□	□	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□				□				□				□
	200	□	□	□	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□				□				□				□
	300	□	□	□	□	□	□	□	□	■	■	■	□	■	■	■	□	■	■	■	□				□				□				□
	400	□	□	□	□	□	□	□	□	□	□	□	□	■	■	■	□	■	■	■	□				□				□				□
Y8 - *Penicillium echinulatum*	0	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	S	S	S	□	S	S	S	□	S	S	S	□
	100	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□				□				□				□
	200	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□				□				□				□
	300	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□				□				□				□
	400	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□				□				□				□
Y4 - *Penicillium glabrum*	0	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	S			□	S	S		□	S	S	S	□
	100	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□				□				□				□
	200	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□				□				□				□
	300	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□				□				□				□
	400	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□				□				□				□
Y16 - *Penicillium glabrum*	0	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□				□				□				□
	100	□	□	□	□	□	□	□	□	■	■	■	□	■	■	■	□	■	■	■	□				□				□				□
	200	□	□	□	□	□	□	□	□	■	■	■	□	■	■	■	□	■	■	■	□				□				□				□
	300	□	□	□	□	□	□	□	□	□	□	□	□	■	■	■	□	■	■	■	□				□				□				□
	400	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	■	■	■	□				□				□				□
Y17 - *Penicillium spinolosum*	0	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	S		S	□	S		S	□	S		S	□
	100	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□				□				□				□
	200	□	□	□	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□				□				□				□
	300	□	□	□	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□				□				□				□
	400	□	□	□	□	□	□	□	□	■	■	■	□	■	■	■	□	■	■	■	□				□				□				□
Y7 - *Penicillium verucosum**	0	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□				□				□				□
	100	□	□	□	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□				□				□				□
	200	□	□	□	□	■	■	■	□	■	■	■	□	■	■	■	□	■	■	■	□				□				□				□
	300	□	□	□	□	□	□	□	□	■	■	■	□	■	■	■	□	■	■	■	□				□				□				□
	400	□	□	□	□	□	□	□	□	□	□	□	□	■	■	■	□	■	■	■	□				□				□				□

Figure A.10 - Detailed observations over 15 days, of the effect of potassium sorbate on filamentous fungi. S1, S2 and S3 represent each strain replicate and MC represents the media sterility control. ■ - Observable Growth, □ – No Observable Growth, S- Observable Conidia Formation *- Mycotoxigenic Strain

Appendix

A.3 Effect of mixture of preservative/weak organic acid solutions on the growth boundary of spoilage fungi.

Yeast Strains	PPM	Visual examination scheme (days)											
		1											
		S1				S2				S3			
	|S| \ |B|	50	100	150	200	50	100	150	200	50	100	150	200
Candida albicans	100												
	200												
	300												
	400												
Candida parapsilosis	100												
	200												
	300												
	400												
Candida tropicalis	100												
	200												
	300												
	400												
Clavispora lusitaniae	100												
	200												
	300												
	400												
Pichia membranifaciens	100												
	200												
	300												
	400												
Saccharomyces cerevisiae	100												
	200												
	300												
	400												
Zygosaccharomyces bailii	100												
	200												
	300												
	400												
Negative Control	100												
	200												
	300												
	400												

Yeast Strains	PPM	2											
		S1				S2				S3			
	|S| \ |B|	50	100	150	200	50	100	150	200	50	100	150	200
Candida albicans	100												
	200												
	300												
	400												
Candida parapsilosis	100												
	200												
	300												
	400												
Candida tropicalis	100												
	200												
	300												
	400												
Clavispora lusitaniae	100												
	200												
	300												
	400												
Pichia membranifaciens	100												
	200												
	300												
	400												
Saccharomyces cerevisiae	100												
	200												
	300												
	400												
Zygosaccharomyces bailii	100												
	200												
	300												
	400												
Negative Control	100												
	200												
	300												
	400												

Yeast Strains	PPM	3											
		S1				S2				S3			
	|S| \ |B|	50	100	150	200	50	100	150	200	50	100	150	200
Candida albicans	100												
	200												
	300												
	400												
Candida parapsilosis	100												
	200												
	300												
	400												
Candida tropicalis	100												
	200												
	300												
	400												
Clavispora lusitaniae	100												
	200												
	300												
	400												
Pichia membranifaciens	100												
	200												
	300												
	400												
Saccharomyces cerevisiae	100												
	200												
	300												
	400												
Zygosaccharomyces bailii	100												
	200												
	300												
	400												
Negative Control	100												
	200												
	300												
	400												

Yeast Strains	PPM	4											
		S1				S2				S3			
	|S| \ |B|	50	100	150	200	50	100	150	200	50	100	150	200
Candida albicans	100												
	200												
	300												
	400												
Candida parapsilosis	100												
	200												
	300												
	400												
Candida tropicalis	100												
	200												
	300												
	400												
Clavispora lusitaniae	100												
	200												
	300												
	400												
Pichia membranifaciens	100												
	200												
	300												
	400												
Saccharomyces cerevisiae	100												
	200												
	300												
	400												
Zygosaccharomyces bailii	100												
	200												
	300												
	400												
Negative Control	100												
	200												
	300												
	400												

Yeast Strains	PPM	5											
		S1				S2				S3			
	|S| \ |B|	50	100	150	200	50	100	150	200	50	100	150	200
Candida albicans	100												
	200												
	300												
	400												
Candida parapsilosis	100												
	200												
	300												
	400												
Candida tropicalis	100												
	200												
	300												
	400												
Clavispora lusitaniae	100												
	200												
	300												
	400												
Pichia membranifaciens	100												
	200												
	300												
	400												
Saccharomyces cerevisiae	100												
	200												
	300												
	400												
Zygosaccharomyces bailii	100												
	200												
	300												
	400												
Negative Control	100												
	200												
	300												
	400												

Figure A.11 - Detailed observations of the first 5 days, of the effect of sodium benzoate and potassium sorbate mixture on yeasts. S1, S2 and S3 represent each strain replicate and negative control represents the media sterility control. ■ - Observable Growth, □ – No Observable Growth

Yeast Strains	PPM	Visual examination scheme (days)																																											
		7												11												15												21							
	B / S	S1				S2				S3				S1				S2				S3				S1				S2				S3				S1				S2			
		50	100	150	200	50	100	150	200	50	100	150	200	50	100	150	200	50	100	150	200	50	100	150	200	50	100	150	200	50	100	150	200	50	100	150	200	50	100	150	200	50	100	150	200
Candida albicans	100																																												
	200																																												
	300																																												
	400																																												
Candida parapsilosis	100																																												
	200																																												
	300																																												
	400																																												
Candida tropicalis	100																																												
	200																																												
	300																																												
	400																																												
Clavispora lusitaniae	100																																												
	200																																												
	300																																												
	400																																												
Pichia membranifaciens	100																																												
	200																																												
	300																																												
	400																																												
Saccharomyces cerevisiae	100																																												
	200																																												
	300																																												
	400																																												
Zygosaccharomyces bailii	100																																												
	200																																												
	300																																												
	400																																												
Negative Control	100																																												
	200																																												
	300																																												
	400																																												

Figure A.12 - Detailed observations of the last 14 days, of the effect of sodium benzoate and potassium sorbate mixture on yeasts. S1, S2 and S3 represent each strain replicate and negative control represents the media sterility control. ■ - Observable Growth, □ – No Observable Growth

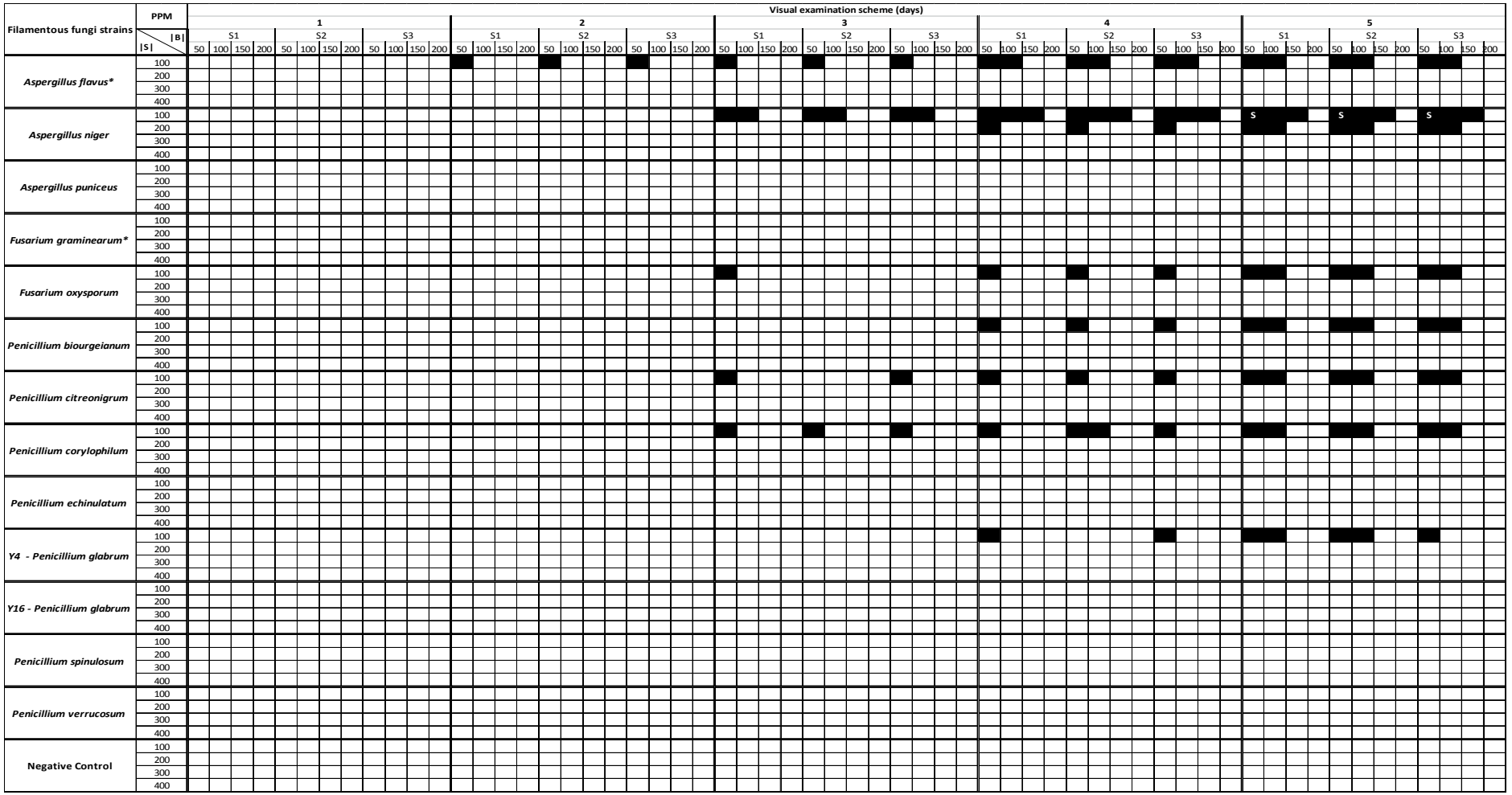

Figure A.13 - Detailed observations of the first 5 days, of the effect of sodium benzoate and potassium sorbate mixture on filamentous fungi. S1, S2 and S3 represent each strain replicate and negative control represents the media sterility control. ■ - Observable Growth, □ – No Observable Growth, S - Observable Conidia Formation, *-Mycotoxigenic Strain

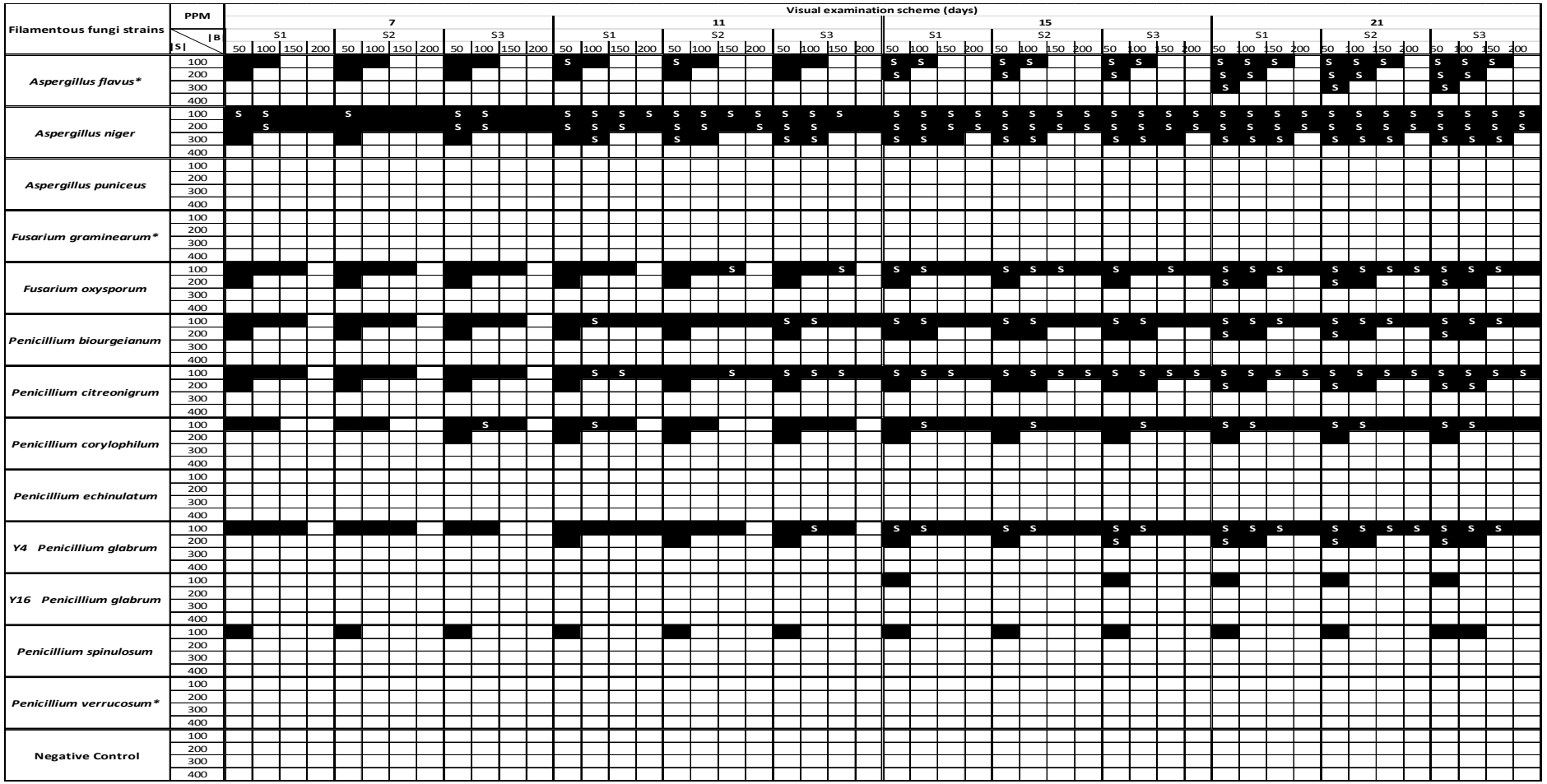

Figure A.14 - Detailed observations of the last 14 days, of the effect of sodium benzoate and potassium sorbate mixture on filamentous fungi. S1, S2 and S3 represent each strain replicate and negative control represents the media sterility control. ■ - Observable Growth, □ – No Observable Growth, S - Observable Conidia Formation, *-Mycotoxigenic Strain

Yeast Strains	PPM	Visual examination Scheme (days): 1											
	|F| |S|	S1				S2				S3			
		125	250	500	1000	125	250	500	1000	125	250	500	1000
Candida albicans	100												
	200												
	300												
	400												
Candida parapsilosis	100												
	200												
	300												
	400												
Candida tropicalis	100												
	200												
	300												
	400												
Clavispora lusitaniae	100												
	200												
	300												
	400												
Pichia membranifaciens	100												
	200												
	300												
	400												
Saccharomyces cerevisiae	100												
	200												
	300												
	400												
Zygosaccharomyces bailii	100												
	200												
	300												
	400												
Negative Control	100												
	200												
	300												
	400												

Yeast Strains	PPM	Visual examination Scheme (days): 2											
	|F| |S|	S1				S2				S3			
		125	250	500	1000	125	250	500	1000	125	250	500	1000
Candida albicans	100												
	200												
	300												
	400												
Candida parapsilosis	100												
	200												
	300												
	400												
Candida tropicalis	100												
	200												
	300												
	400												
Clavispora lusitaniae	100												
	200												
	300												
	400												
Pichia membranifaciens	100												
	200												
	300												
	400												
Saccharomyces cerevisiae	100												
	200												
	300												
	400												
Zygosaccharomyces bailii	100												
	200												
	300												
	400												
Negative Control	100												
	200												
	300												
	400												

Yeast Strains	PPM	Visual examination Scheme (days): 3											
	|F| |S|	S1				S2				S3			
		125	250	500	1000	125	250	500	1000	125	250	500	1000
Candida albicans	100												
	200												
	300												
	400												
Candida parapsilosis	100												
	200												
	300												
	400												
Candida tropicalis	100												
	200												
	300												
	400												
Clavispora lusitaniae	100												
	200												
	300												
	400												
Pichia membranifaciens	100	■	■	■	■	■	■	■	■	■	■	■	■
	200												
	300												
	400												
Saccharomyces cerevisiae	100												
	200												
	300												
	400												
Zygosaccharomyces bailii	100	■	■	■	■	■	■	■	■	■	■	■	■
	200												
	300												
	400												
Negative Control	100												
	200												
	300												
	400												

Yeast Strains	PPM	Visual examination Scheme (days): 4											
	|F| |S|	S1				S2				S3			
		125	250	500	1000	125	250	500	1000	125	250	500	1000
Candida albicans	100												
	200												
	300												
	400												
Candida parapsilosis	100												
	200												
	300												
	400												
Candida tropicalis	100												
	200												
	300												
	400												
Clavispora lusitaniae	100												
	200												
	300												
	400												
Pichia membranifaciens	100	■	■	■	■	■	■	■	■	■	■	■	■
	200												
	300												
	400												
Saccharomyces cerevisiae	100												
	200												
	300												
	400												
Zygosaccharomyces bailii	100	■	■	■	■	■	■	■	■	■	■	■	■
	200												
	300												
	400												
Negative Control	100												
	200												
	300												
	400												

Figure A.15 - Detailed observations of the first 5 days, of the effect of potassium sorbate and fumaric acid mixture on yeasts. S1, S2 and S3 represent each strain replicate and negative control represents the media sterility control. ■ - Observable Growth, □ – No Observable Growth

Yeast Strains	PPM |S| \ |F|	Visual examination Scheme (days)																																											
		5													7												10												15									
		S1				S2				S3				S1				S2				S3				S1				S2				S3				S1				S2				S3			
		125	250	500	1000	125	250	500	1000	125	250	500	1000	125	250	500	1000	125	250	500	1000	125	250	500	1000	125	250	500	1000	125	250	500	1000	125	250	500	1000	125	250	500	1000	125	250	500	1000	125	250	500	1000
Candida albicans	100																																														
	200																																														
	300																																														
	400																																														
Candida parapsilosis	100																																														
	200																																														
	300																																														
	400																																														
Candida tropicalis	100																																														
	200																																														
	300																																														
	400																																														
Clavispora lusitaniae	100																																														
	200																																														
	300																																														
	400																																														
Pichia membranifaciens	100																																														
	200																																														
	300																																														
	400																																														
Saccharomyces cerevisiae	100																																														
	200																																														
	300																																														
	400																																														
Zygosaccharomyces bailii	100																																														
	200																																														
	300																																														
	400																																														
Negative Control	100																																													
	200																																														
	300																																														
	400																																														

Figure A.16 - Detailed observations of the last 14 days, of the effect of potassium sorbate and fumaric acid mixture on yeasts. S1, S2 and S3 represent each strain replicate and negative control represents the media sterility control. ■ - Observable Growth, □ – No Observable Growth

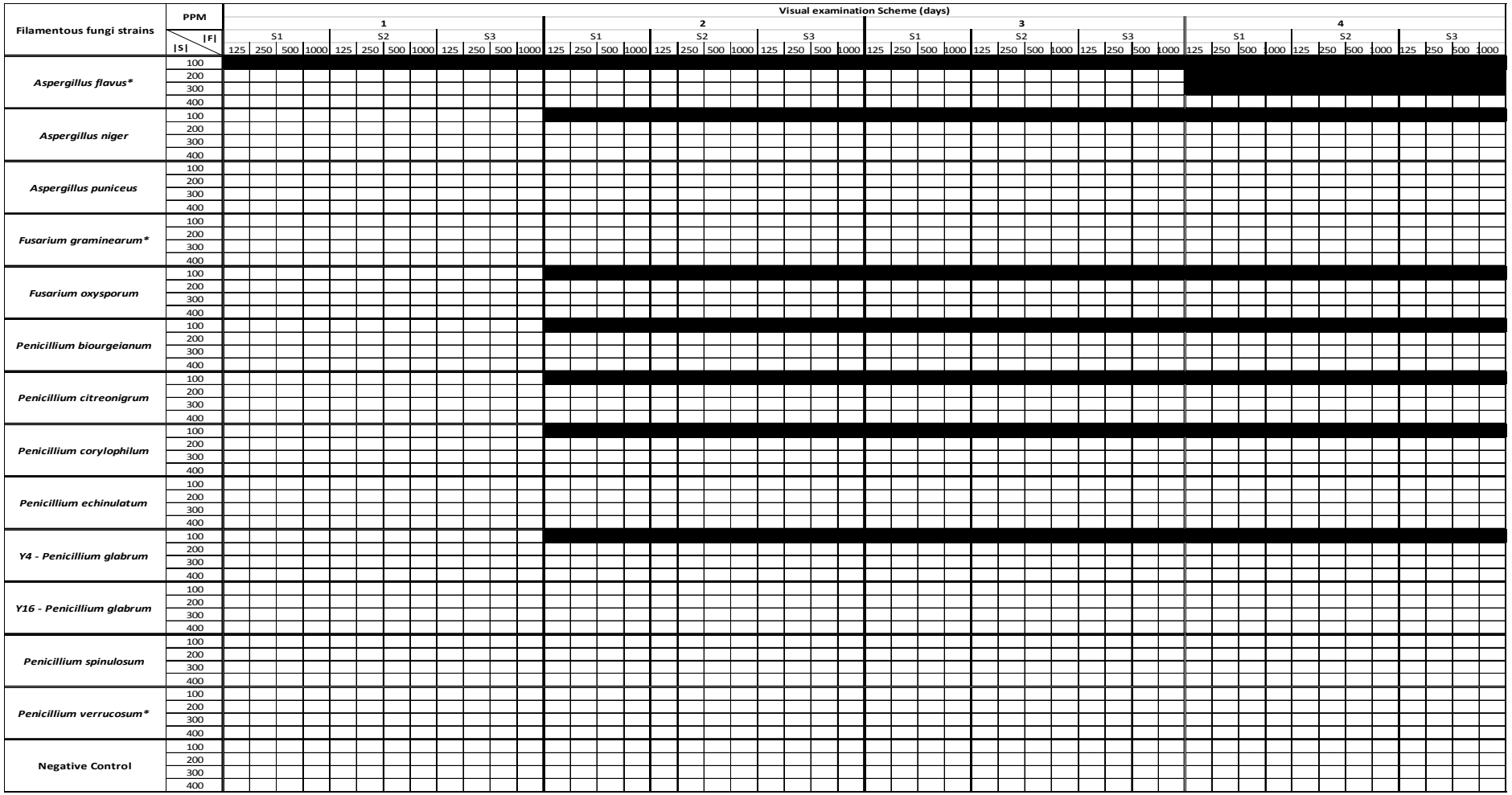

Figure A.17 - Detailed observations of the first 5 days, of the effect of potassium sorbate and fumaric acid mixture on filamentous fungi. S1, S2 and S3 represent each strain replicate and negative control represents the media sterility control. ■ - Observable Growth, □ – No Observable Growth, S - Observable Conidia Formation, *-Mycotoxigenic Strain

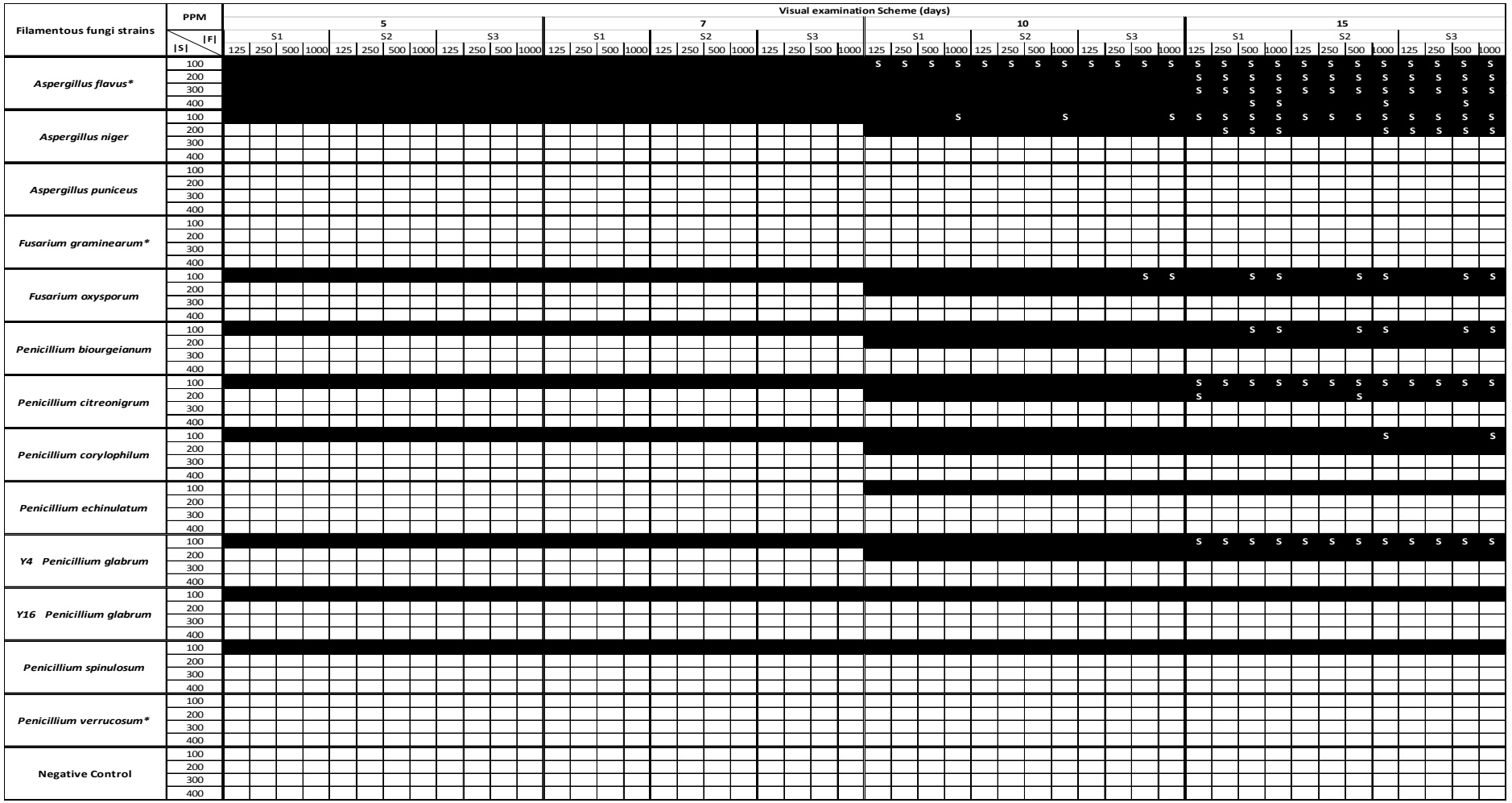

Figure A.18 - Detailed observations of the last 14 days, of the effect of potassium sorbate and fumaric acid mixture on filamentous fungi. S1, S2 and S3 represent each strain replicate and negative control represents the media sterility control. ■ - Observable Growth, □ – No Observable Growth, S - Observable Conidia Formation, *-Mycotoxigenic Strain

Yeast Strains	PPM	Visual examination Scheme (days)																																															
		1												2												3												4											
		S1				S2				S3				S1				S2				S3				S1				S2				S3				S1				S2				S3			
	|M| |S|	125	250	500	1000	125	250	500	1000	125	250	500	1000	125	250	500	1000	125	250	500	1000	125	250	500	1000	125	250	500	1000	125	250	500	1000	125	250	500	1000	125	250	500	1000	125	250	500	1000	125	250	500	1000
Candida albicans	100																																																
	200																																																
	300																																																
	400																																																
Candida parapsilosis	100																																																
	200																																																
	300																																																
	400																																																
Candida tropicalis	100																																																
	200																																																
	300																																																
	400																																																
Clavispora lusitaniae	100																																																
	200																																																
	300																																																
	400																																																
Pichia membranifaciens	100																																																
	200																																																
	300																																																
	400																																																
Saccharomyces cerevisiae	100																																																
	200																																																
	300																																																
	400																																																
Zygosaccharomyces bailii	100																																																
	200																																																
	300																																																
	400																																																
Negative Control	100																																																
	200																																																
	300																																																
	400																																																

Figure A.19 - Detailed observations of the first 5 days, of the effect of potassium sorbate and malic acid mixture on yeasts. S1, S2 and S3 represent each strain replicate and negative control represents the media sterility control. ■ - Observable Growth, □ – No Observable Growth

Yeast Strains	PPM |S| / |M|	Visual examination Scheme (days)																																					
		5												7												10								15							
		S1				S2				S3				S1				S2				S3				S1				S2				S3				S1			
		125	250	500	1000	125	250	500	1000	125	250	500	1000	125	250	500	1000	125	250	500	1000	125	250	500	1000	125	250	500	1000	125	250	500	1000	125	250	500	1000	125	250	500	1000
Candida albicans	100																																							
	200																																							
	300																																							
	400																																							
Candida parapsilosis	100																																							
	200																																							
	300																																							
	400																																							
Candida tropicalis	100																																							
	200																																							
	300																																							
	400																																							
Clavispora lusitaniae	100																																							
	200																																							
	300																																							
	400																																							
Pichia membranifaciens	100																									■				■				■				■	■	■	■
	200																																							
	300																																							
	400																																							
Saccharomyces cerevisiae	100																																							
	200																																							
	300																																							
	400																																							
Zygosaccharomyces bailii	100																									■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■
	200																																							
	300																																							
	400																																							
Negative Control	100																																							
	200																																							
	300																																							
	400																																							

Figure A.20 - Detailed observations of the last 14 days, of the effect of potassium sorbate and malic acid mixture on yeasts. S1, S2 and S3 represent each strain replicate and negative control represents the media sterility control. ■ - Observable Growth, □ – No Observable Growth

Filamentous fungi strains	PPM |S| \ |M|	Day 1 S1 125	S1 250	S1 500	S1 1000	S2 125	S2 250	S2 500	S2 1000	S3 125	S3 250	S3 500	S3 1000
*Aspergillus flavus**	100	■	■	■	■	■	■	■	■	■	■	■	■
	200	■	□	□	□	■	□	□	□	■	□	□	□
	300	□	□	□	□	□	□	□	□	□	□	□	□
	400	□	□	□	□	□	□	□	□	□	□	□	□
Aspergillus niger	100	□	□	□	□	□	□	□	□	□	□	□	□
	200	□	□	□	□	□	□	□	□	□	□	□	□
	300	□	□	□	□	□	□	□	□	□	□	□	□
	400	□	□	□	□	□	□	□	□	□	□	□	□
Aspergillus puniceus	100	□	□	□	□	□	□	□	□	□	□	□	□
	200	□	□	□	□	□	□	□	□	□	□	□	□
	300	□	□	□	□	□	□	□	□	□	□	□	□
	400	□	□	□	□	□	□	□	□	□	□	□	□
*Fusarium graminearum**	100	□	□	□	□	□	□	□	□	□	□	□	□
	200	□	□	□	□	□	□	□	□	□	□	□	□
	300	□	□	□	□	□	□	□	□	□	□	□	□
	400	□	□	□	□	□	□	□	□	□	□	□	□
Fusarium oxysporum	100	□	□	□	□	□	□	□	□	□	□	□	□
	200	□	□	□	□	□	□	□	□	□	□	□	□
	300	□	□	□	□	□	□	□	□	□	□	□	□
	400	□	□	□	□	□	□	□	□	□	□	□	□
Penicillium biourgeianum	100	□	□	□	□	□	□	□	□	□	□	□	□
	200	□	□	□	□	□	□	□	□	□	□	□	□
	300	□	□	□	□	□	□	□	□	□	□	□	□
	400	□	□	□	□	□	□	□	□	□	□	□	□
Penicillium citreonigrum	100	□	□	□	□	□	□	□	□	□	□	□	□
	200	□	□	□	□	□	□	□	□	□	□	□	□
	300	□	□	□	□	□	□	□	□	□	□	□	□
	400	□	□	□	□	□	□	□	□	□	□	□	□
Penicillium corylophilum	100	□	□	□	□	□	□	□	□	□	□	□	□
	200	□	□	□	□	□	□	□	□	□	□	□	□
	300	□	□	□	□	□	□	□	□	□	□	□	□
	400	□	□	□	□	□	□	□	□	□	□	□	□
Penicillium echinulatum	100	□	□	□	□	□	□	□	□	□	□	□	□
	200	□	□	□	□	□	□	□	□	□	□	□	□
	300	□	□	□	□	□	□	□	□	□	□	□	□
	400	□	□	□	□	□	□	□	□	□	□	□	□
Y4 - Penicillium glabrum	100	□	□	□	□	□	□	□	□	□	□	□	□
	200	□	□	□	□	□	□	□	□	□	□	□	□
	300	□	□	□	□	□	□	□	□	□	□	□	□
	400	□	□	□	□	□	□	□	□	□	□	□	□
Y16 - Penicillium glabrum	100	□	□	□	□	□	□	□	□	□	□	□	□
	200	□	□	□	□	□	□	□	□	□	□	□	□
	300	□	□	□	□	□	□	□	□	□	□	□	□
	400	□	□	□	□	□	□	□	□	□	□	□	□
Penicillium spinulosum	100	□	□	□	□	□	□	□	□	□	□	□	□
	200	□	□	□	□	□	□	□	□	□	□	□	□
	300	□	□	□	□	□	□	□	□	□	□	□	□
	400	□	□	□	□	□	□	□	□	□	□	□	□
*Penicillium verrucosum**	100	□	□	□	□	□	□	□	□	□	□	□	□
	200	□	□	□	□	□	□	□	□	□	□	□	□
	300	□	□	□	□	□	□	□	□	□	□	□	□
	400	□	□	□	□	□	□	□	□	□	□	□	□
Negative Control	100	□	□	□	□	□	□	□	□	□	□	□	□
	200	□	□	□	□	□	□	□	□	□	□	□	□
	300	□	□	□	□	□	□	□	□	□	□	□	□
	400	□	□	□	□	□	□	□	□	□	□	□	□

Filamentous fungi strains	PPM |S| \ |M|	Day 2 S1 125	S1 250	S1 500	S1 1000	S2 125	S2 250	S2 500	S2 1000	S3 125	S3 250	S3 500	S3 1000
*Aspergillus flavus**	100	■	■	■	■	■	■	■	■	■	■	■	■
	200	■	□	□	□	■	□	□	□	■	□	□	□
	300	□	□	□	□	□	□	□	□	□	□	□	□
	400	□	□	□	□	□	□	□	□	□	□	□	□
Aspergillus niger	100	■	■	■	■	■	■	■	■	■	■	■	■
	200	□	□	□	□	□	□	□	□	□	□	□	□
	300	□	□	□	□	□	□	□	□	□	□	□	□
	400	□	□	□	□	□	□	□	□	□	□	□	□
Aspergillus puniceus	100	□	□	□	□	□	□	□	□	□	□	□	□
	200	□	□	□	□	□	□	□	□	□	□	□	□
	300	□	□	□	□	□	□	□	□	□	□	□	□
	400	□	□	□	□	□	□	□	□	□	□	□	□
*Fusarium graminearum**	100	□	□	□	□	□	□	□	□	□	□	□	□
	200	□	□	□	□	□	□	□	□	□	□	□	□
	300	□	□	□	□	□	□	□	□	□	□	□	□
	400	□	□	□	□	□	□	□	□	□	□	□	□
Fusarium oxysporum	100	■	■	■	■	■	■	■	■	■	■	■	■
	200	□	□	□	□	□	□	□	□	□	□	□	□
	300	□	□	□	□	□	□	□	□	□	□	□	□
	400	□	□	□	□	□	□	□	□	□	□	□	□
Penicillium biourgeianum	100	■	■	■	■	■	■	■	■	■	■	■	■
	200	□	□	□	□	□	□	□	□	□	□	□	□
	300	□	□	□	□	□	□	□	□	□	□	□	□
	400	□	□	□	□	□	□	□	□	□	□	□	□
Penicillium citreonigrum	100	■	■	■	■	■	■	■	■	■	■	■	■
	200	□	□	□	□	□	□	□	□	□	□	□	□
	300	□	□	□	□	□	□	□	□	□	□	□	□
	400	□	□	□	□	□	□	□	□	□	□	□	□
Penicillium corylophilum	100	□	□	□	□	□	□	□	□	□	□	□	□
	200	□	□	□	□	□	□	□	□	□	□	□	□
	300	□	□	□	□	□	□	□	□	□	□	□	□
	400	□	□	□	□	□	□	□	□	□	□	□	□
Penicillium echinulatum	100	□	□	□	□	□	□	□	□	□	□	□	□
	200	□	□	□	□	□	□	□	□	□	□	□	□
	300	□	□	□	□	□	□	□	□	□	□	□	□
	400	□	□	□	□	□	□	□	□	□	□	□	□
Y4 - Penicillium glabrum	100	■	■	■	■	■	■	■	■	■	■	■	■
	200	□	□	□	□	□	□	□	□	□	□	□	□
	300	□	□	□	□	□	□	□	□	□	□	□	□
	400	□	□	□	□	□	□	□	□	□	□	□	□
Y16 - Penicillium glabrum	100	□	□	□	□	□	□	□	□	□	□	□	□
	200	□	□	□	□	□	□	□	□	□	□	□	□
	300	□	□	□	□	□	□	□	□	□	□	□	□
	400	□	□	□	□	□	□	□	□	□	□	□	□
Penicillium spinulosum	100	□	□	□	□	□	□	□	□	□	□	□	□
	200	□	□	□	□	□	□	□	□	□	□	□	□
	300	□	□	□	□	□	□	□	□	□	□	□	□
	400	□	□	□	□	□	□	□	□	□	□	□	□
*Penicillium verrucosum**	100	□	□	□	□	□	□	□	□	□	□	□	□
	200	□	□	□	□	□	□	□	□	□	□	□	□
	300	□	□	□	□	□	□	□	□	□	□	□	□
	400	□	□	□	□	□	□	□	□	□	□	□	□
Negative Control	100	□	□	□	□	□	□	□	□	□	□	□	□
	200	□	□	□	□	□	□	□	□	□	□	□	□
	300	□	□	□	□	□	□	□	□	□	□	□	□
	400	□	□	□	□	□	□	□	□	□	□	□	□

Filamentous fungi strains	PPM |S| \ |M|	Day 3 S1 125	S1 250	S1 500	S1 1000	S2 125	S2 250	S2 500	S2 1000	S3 125	S3 250	S3 500	S3 1000
*Aspergillus flavus**	100	■	■	■	■	■	■	■	■	■	■	■	■
	200	■	□	□	□	■	□	□	□	■	□	□	□
	300	□	□	□	□	□	□	□	□	□	□	□	□
	400	□	□	□	□	□	□	□	□	□	□	□	□
Aspergillus niger	100	■	■	■	■	■	■	■	■	■	■	■	■
	200	□	□	□	□	□	□	□	□	□	□	□	□
	300	□	□	□	□	□	□	□	□	□	□	□	□
	400	□	□	□	□	□	□	□	□	□	□	□	□
Aspergillus puniceus	100	□	□	□	□	□	□	□	□	□	□	□	□
	200	□	□	□	□	□	□	□	□	□	□	□	□
	300	□	□	□	□	□	□	□	□	□	□	□	□
	400	□	□	□	□	□	□	□	□	□	□	□	□
*Fusarium graminearum**	100	□	□	□	□	□	□	□	□	□	□	□	□
	200	□	□	□	□	□	□	□	□	□	□	□	□
	300	□	□	□	□	□	□	□	□	□	□	□	□
	400	□	□	□	□	□	□	□	□	□	□	□	□
Fusarium oxysporum	100	■	■	■	■	■	■	■	■	■	■	■	■
	200	□	□	□	□	□	□	□	□	□	□	□	□
	300	□	□	□	□	□	□	□	□	□	□	□	□
	400	□	□	□	□	□	□	□	□	□	□	□	□
Penicillium biourgeianum	100	■	■	■	■	■	■	■	■	■	■	■	■
	200	□	□	□	□	□	□	□	□	□	□	□	□
	300	□	□	□	□	□	□	□	□	□	□	□	□
	400	□	□	□	□	□	□	□	□	□	□	□	□
Penicillium citreonigrum	100	■	■	■	■	■	■	■	■	■	■	■	■
	200	□	□	□	□	□	□	□	□	□	□	□	□
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	400	□	□	□	□	□	□	□	□	□	□	□	□
Penicillium corylophilum	100	■	■	■	■	■	■	■	■	■	■	■	■
	200	□	□	□	□	□	□	□	□	□	□	□	□
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	400	□	□	□	□	□	□	□	□	□	□	□	□
Penicillium echinulatum	100	□	□	□	□	□	□	□	□	□	□	□	□
	200	□	□	□	□	□	□	□	□	□	□	□	□
	300	□	□	□	□	□	□	□	□	□	□	□	□
	400	□	□	□	□	□	□	□	□	□	□	□	□
Y4 - Penicillium glabrum	100	■	■	■	■	■	■	■	■	■	■	■	■
	200	□	□	□	□	□	□	□	□	□	□	□	□
	300	□	□	□	□	□	□	□	□	□	□	□	□
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Y16 - Penicillium glabrum	100	□	□	□	□	□	□	□	□	□	□	□	□
	200	□	□	□	□	□	□	□	□	□	□	□	□
	300	□	□	□	□	□	□	□	□	□	□	□	□
	400	□	□	□	□	□	□	□	□	□	□	□	□
Penicillium spinulosum	100	□	□	□	□	□	□	□	□	□	□	□	□
	200	□	□	□	□	□	□	□	□	□	□	□	□
	300	□	□	□	□	□	□	□	□	□	□	□	□
	400	□	□	□	□	□	□	□	□	□	□	□	□
*Penicillium verrucosum**	100	□	□	□	□	□	□	□	□	□	□	□	□
	200	□	□	□	□	□	□	□	□	□	□	□	□
	300	□	□	□	□	□	□	□	□	□	□	□	□
	400	□	□	□	□	□	□	□	□	□	□	□	□
Negative Control	100	□	□	□	□	□	□	□	□	□	□	□	□
	200	□	□	□	□	□	□	□	□	□	□	□	□
	300	□	□	□	□	□	□	□	□	□	□	□	□
	400	□	□	□	□	□	□	□	□	□	□	□	□

Filamentous fungi strains	PPM |S| \ |M|	Day 4 S1 125	S1 250	S1 500	S1 1000	S2 125	S2 250	S2 500	S2 1000	S3 125	S3 250	S3 500	S3 1000
*Aspergillus flavus**	100	■	■	■	■	■	■	■	■	■	■	■	■
	200	■	■	■	■	■	■	■	■	■	■	■	■
	300	■	■	■	■	■	■	■	■	■	■	■	■
	400	□	□	□	□	□	□	□	□	□	□	□	□
Aspergillus niger	100	■	■	■	■	■	■	■	■	■	■	■	■
	200	□	□	□	□	□	□	□	□	□	□	□	□
	300	□	□	□	□	□	□	□	□	□	□	□	□
	400	□	□	□	□	□	□	□	□	□	□	□	□
Aspergillus puniceus	100	□	□	□	□	□	□	□	□	□	□	□	□
	200	□	□	□	□	□	□	□	□	□	□	□	□
	300	□	□	□	□	□	□	□	□	□	□	□	□
	400	□	□	□	□	□	□	□	□	□	□	□	□
*Fusarium graminearum**	100	□	□	□	□	□	□	□	□	□	□	□	□
	200	□	□	□	□	□	□	□	□	□	□	□	□
	300	□	□	□	□	□	□	□	□	□	□	□	□
	400	□	□	□	□	□	□	□	□	□	□	□	□
Fusarium oxysporum	100	■	■	■	■	■	■	■	■	■	■	■	■
	200	□	□	□	□	□	□	□	□	□	□	□	□
	300	□	□	□	□	□	□	□	□	□	□	□	□
	400	□	□	□	□	□	□	□	□	□	□	□	□
Penicillium biourgeianum	100	■	■	■	■	■	■	■	■	■	■	■	■
	200	□	□	□	□	□	□	□	□	□	□	□	□
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	400	□	□	□	□	□	□	□	□	□	□	□	□
Penicillium citreonigrum	100	■	■	■	■	■	■	■	■	■	■	■	■
	200	□	□	□	□	□	□	□	□	□	□	□	□
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	400	□	□	□	□	□	□	□	□	□	□	□	□
Penicillium corylophilum	100	■	■	■	■	■	■	■	■	■	■	■	■
	200	□	□	□	□	□	□	□	□	□	□	□	□
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	400	□	□	□	□	□	□	□	□	□	□	□	□
Penicillium echinulatum	100	□	□	□	□	□	□	□	□	□	□	□	□
	200	□	□	□	□	□	□	□	□	□	□	□	□
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	400	□	□	□	□	□	□	□	□	□	□	□	□
Y4 - Penicillium glabrum	100	■	■	■	■	■	■	■	■	■	■	■	■
	200	□	□	□	□	□	□	□	□	□	□	□	□
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	400	□	□	□	□	□	□	□	□	□	□	□	□
Y16 - Penicillium glabrum	100	□	□	□	□	□	□	□	□	□	□	□	□
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	300	□	□	□	□	□	□	□	□	□	□	□	□
	400	□	□	□	□	□	□	□	□	□	□	□	□
Penicillium spinulosum	100	□	□	□	□	□	□	□	□	□	□	□	□
	200	□	□	□	□	□	□	□	□	□	□	□	□
	300	□	□	□	□	□	□	□	□	□	□	□	□
	400	□	□	□	□	□	□	□	□	□	□	□	□
*Penicillium verrucosum**	100	□	□	□	□	□	□	□	□	□	□	□	□
	200	□	□	□	□	□	□	□	□	□	□	□	□
	300	□	□	□	□	□	□	□	□	□	□	□	□
	400	□	□	□	□	□	□	□	□	□	□	□	□
Negative Control	100	□	□	□	□	□	□	□	□	□	□	□	□
	200	□	□	□	□	□	□	□	□	□	□	□	□
	300	□	□	□	□	□	□	□	□	□	□	□	□
	400	□	□	□	□	□	□	□	□	□	□	□	□

Figure A.21 - Detailed observations of the first 5 days, of the effect of potassium sorbate and malic acid mixture on filamentous fungi. S1, S2 and S3 represent each strain replicate and negative control represents the media sterility control. ■ - Observable Growth, □ – No Observable Growth, S - Observable Conidia Formation, *-Mycotoxigenic Strain

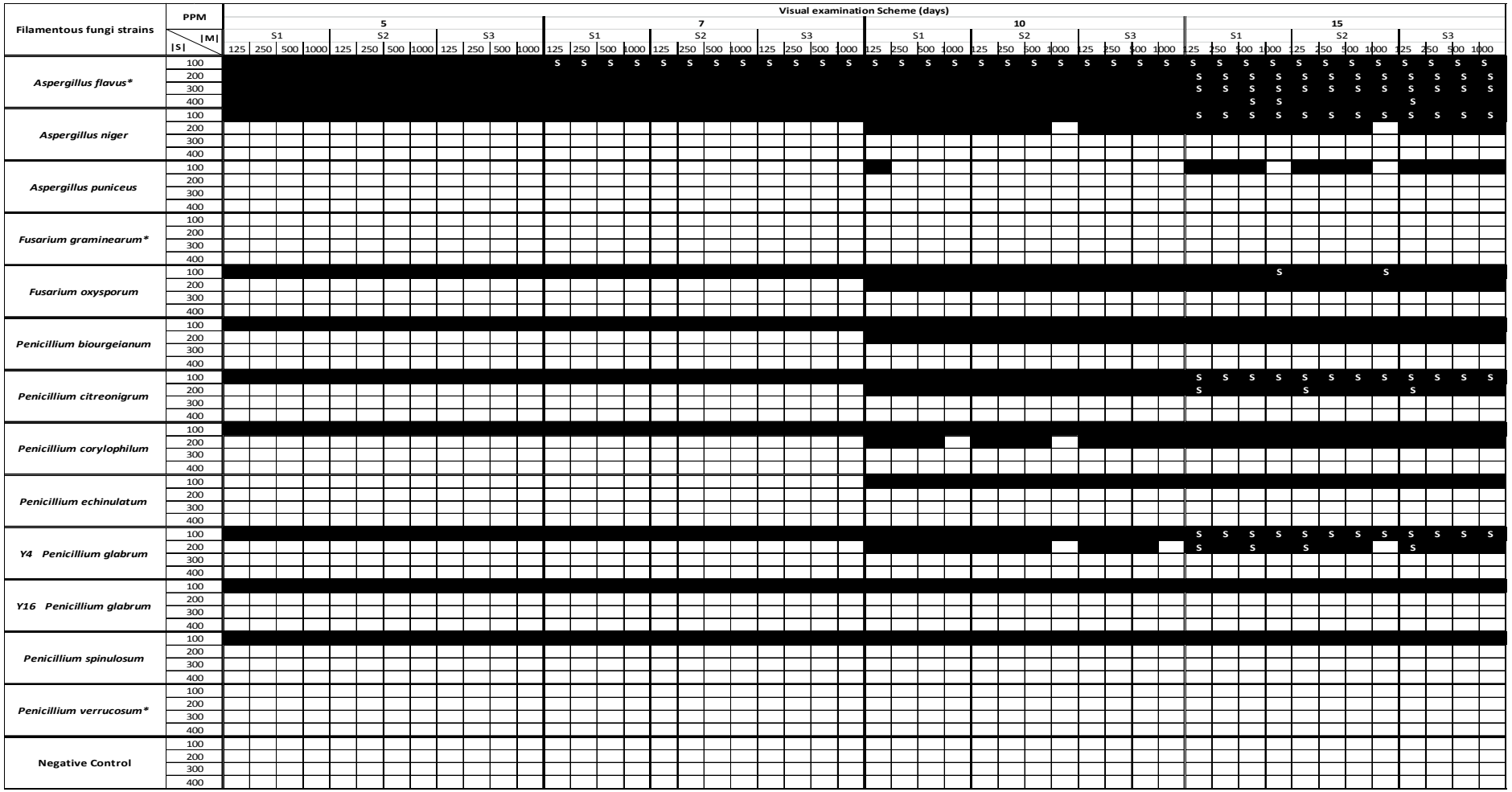

Figure A.22 - Detailed observations of the last 14 days, of the effect of potassium sorbate and malic acid mixture on filamentous fungi. S1, S2 and S3 represent each strain replicate and negative control represents the media sterility control. ■ - Observable Growth, □ – No Observable Growth, S - Observable Conidia Formation, *-Mycotoxigenic Strain

Appendix B

B.1 Peer Reviewed Publications.

Appendix

Journal of Microbiological Methods 88 (2012) 413–418

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Journal of Microbiological Methods

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Comparison of different bead-beating RNA extraction strategies: An optimized method for filamentous fungi

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ABSTRACT

Molecular studies, especially in relation to the activity of secondary metabolite gene clusters, require the ability to extract good quality RNA from fungal biomass. This is often hindered by the cell wall structure and endogenous RNase activity in filamentous fungi. There is thus a need for rapid methods for the extraction of good quality RNA for use in microarrays and for quantitative PCR assays. The objective of this study was to examine the use of different systems for the high throughput method to extract intact RNA from filamentous fungi. Two bead beating systems with different motion patterns and speed capacities were tested in the development of the extraction protocol. They were evaluated based on the total RNA yield and overall RNA quality. The high speed bead beating with glass beads associated with an automated purification method gave more than three times higher total RNA yields with less than a quarter of the amount of mycelium required. Furthermore the integrity and overall quality was conserved, with RNA Quality Indicator (RQI) numbers consistently >7.5. This method also reduced cross contamination risks and kept RNA handling to a minimum while still being capable of multiple sample processing, reducing the time required to obtain RNA from filamentous fungi.

1. Introduction

Rapid advances in molecular biology have promoted the use of molecular techniques in mycological studies. As an example, new research trends with mycotoxigenic fungi have integrated traditional ecological and physiological experiments with molecular data (gene expression and gene switching) to use a system approach to try and provide better insights into the functional role of gene clusters involved in mycotoxin production and a better understanding of fungal behavior, especially in relation to environmental stresses (Abdel-Hadi et al., 2011; Schmidt-Heydt et al., 2009, 2010, 2011).

Thus, the adequate isolation and purification of fungal RNA is a critical step to ensure the successful application of these techniques. The structure of the fungal cell wall makes their disruption for nucleic acid extraction difficult. For this reason the use of standard RNA extraction methods developed for animal cells, bacteria or yeasts cannot be readily applied.

The most common techniques for fungal nucleic acid extraction are the use of cell wall degrading enzymes (Einsele et al., 1997; Williamson et al., 2000) and a physical grinding treatment undertaken with either dry ice or liquid nitrogen (Al-Samarrai and Schmid, 2000; Griffin et al., 2002; Loeffler et al., 2001). For RNA extraction this last physical treatment is the most common methodology applied. It consists of freezing the mycelium with liquid nitrogen and grinding it with the aid of a mortar and a pestle (Abdel-Hadi et al., 2010; Schmidt-Heydt et al., 2007).

A number of studies have reported promising results for mechanical bead-beating extraction procedures to isolate fungal DNA (de Boer et al., 2010; Griffiths et al., 2006; van Burik et al., 1998;), but no special attention has been paid to methods for the isolation of RNA from cultured mycelium, either in liquid or solid agar media. The technique has several advantages over the traditional methods including reduction in time, multiple sample extraction and a reduction of the risk of cross contamination. There is also no need for liquid nitrogen, which is often problematic and hazardous, especially when a large number of samples need to be processed.

To our knowledge, there is no protocol for RNA extraction using any high speed bead-beating method. The aim of this work was to design and develop a bead beating protocol for the extraction of high-quality RNA samples from filamentous fungal biomass. Thus different glass, zirconium oxide, tungsten carbide and stainless steel beads of various sizes were examined in the Precellys 24 homogenizer (Bertin Technologies, Montigny le Bretonneux, France) and in the TissueLyser LT (Qiagen, UK) instruments. Subsequently, the results obtained were compared with those obtained using the traditional method.

2. Materials and methods

2.1. Fungal species and isolates maintenance

An *Aspergillus flavus* strain (NRRL 3357) was used in this study. The strain was maintained on Malt Extract Agar (MEA, Oxoid, UK) at 25 °C. The cultures were subculture on Yeast Extract Sucrose agar

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B.2 Poster Presentations.

Appendix

Cranfield Health

Cranfield UNIVERSITY

The impact of anti-fungal compounds and environmental stress interactions on Fusarium graminearum growth and TRI5 gene expression.

Gonçalo Leite* and Naresh Magan

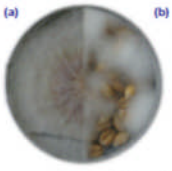
Applied Mycology Group, Cranfield Health, Cranfield, Bedfordshire MK43 0AL, U.K.

Introduction

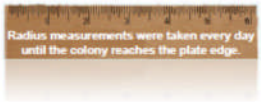
- *Fusarium graminearum* causes ear blight of cereals and can contaminate grain with trichothecenes.
- There are EU regulatory limits for deoxynivalenol (DON) which have to be adhered to (1750 mg/kg in raw cereals).
- *Tri5* encodes trichodiene synthase and is a key regulatory gene in the trichothecene biosynthetic pathway and can be related to DON production.
- Environmental factors(water activity (a_w), temperature) and anti-fungal compounds (antioxidant, fungicide and essential oils) have the capacity to limit *F. graminearum* growth.

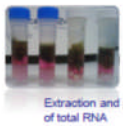
Objectives: This study examined the impact of water stress and anti-fungal compounds on the differential changes in growth and deoxynivalenol production using the *Tri5* gene as a indicator of efficacy.

Methods/Materials

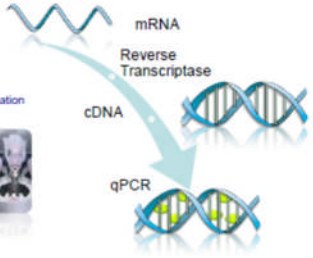


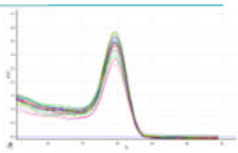
F. graminearum grown on: (a) Glycerol modified Yeast Extract Sucrose agar medium overlaid by cellophane to easily collect the mycelium, (b) gamma irradiated wheat. All study conditions are presented on Table 1.



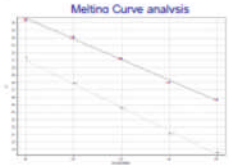


Extraction and purification of total RNA





Melting Curve analysis



Standard curves used to calculate amplification efficiency for each gene (*Tri5* and *β-tubulin* as reference gene) for relative quantification using the Pfaffl method.

Table 1. Compounds and conditions used in this study.

Compound	Glycerol Modified a_w	Temperature (°C)
Control	0.98	25 20 15
Prochloraz	0.96	25
Oil of Thyme		
Butylated hydroxyanisole (BHA)	0.94	

Results

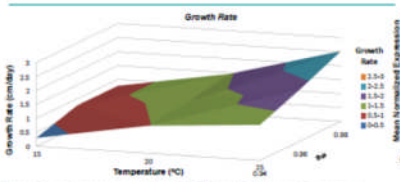


Fig 1. *F. graminearum* growth rate in relation to different a_w versus temperature conditions

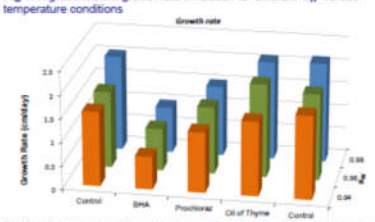


Fig 2. Influence of anti-fungal compounds on growth of *F. graminearum* under different water stress conditions

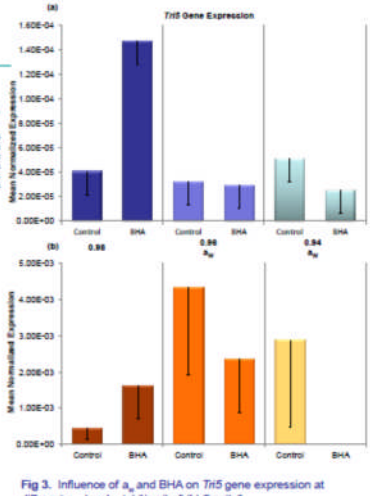


Fig 3. Influence of a_w and BHA on *Tri5* gene expression at different a_w levels. (a) *"in vitro" (b) "in situ"*

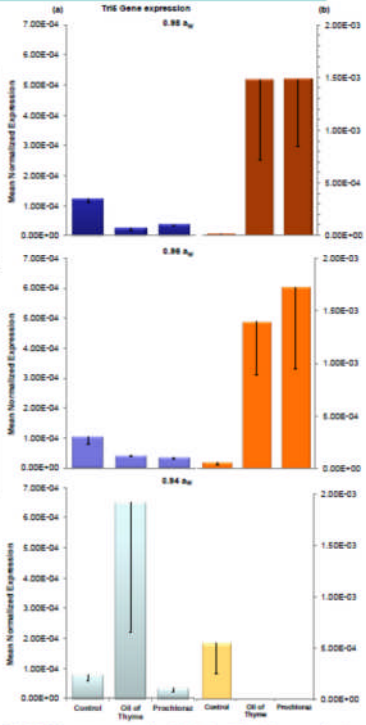


Fig 4. *Tri5* gene expression in relation to antifungal compounds at three different a_w levels. (a) *"in vitro" (b) "in situ"*

Conclusions

- Growth rate and *Tri5 gene expression* were affected by both anti-fungal compounds and the environmental factors.
- *Tri5* gene expression was not directly correlated with growth rate and *"in vitro"* expression levels were not directly correlated with *"in situ"*.
- Prochloraz was the only compound able to reduce both growth rate and *Tri5* gene expression at all water stress conditions and only *"in vitro"*.
- *Tri5* gene expression suggests that DON production can be enhanced by some anti-fungal compounds depending on the water stress imposed.

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The impact of interactions between anti-fungal compounds and environmental stress on growth of *Fusarium graminearum* and deoxynivalenol production using the *TRI5* gene

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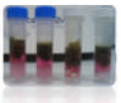
Introduction

- *Fusarium graminearum* causes ear blight of cereals and can contaminate grain with trichothecenes.
- There are EU regulatory limits for deoxynivalenol (DON) which have to be adhered to (1750 mg/kg in raw cereals)
- *Tri5* encodes trichodiene synthase and is a key regulatory gene in the trichothecene biosynthetic pathway and can be related to DON production.
- Several environmental (a_w, temperature) and anti-fungal compounds (antioxidant, fungicide and essential oil) have the capacity to limit *F. graminearum* growth.

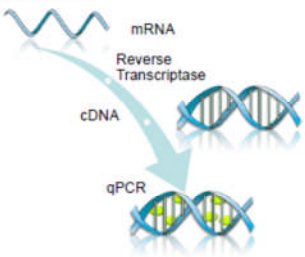
Objectives: This study examined the impact of water stress and anti-fungal compounds on the differential changes in growth and deoxynivalenol production using the *Tri5* gene as a indicator of efficacy.

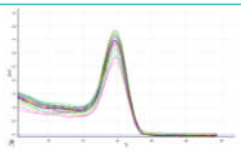
Methods/Materials

F. graminearum grown on Glycerol modified Yeast Extract Sucrose agar medium overlaid by cellophane to easily collect the mycelium. All study conditions are presented on table 1.

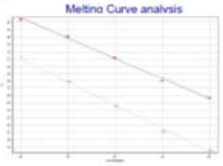


Extraction and purification of total RNA





Melting Curve analysis



Standard curves used to calculate amplification efficiency for each gene (*Tri5* and *β-tubulin* as reference gene) for relative quantification using the Pfaffl method.

Table 1. Compounds and conditions used in this study.

Compound	Final concentration	Glycerol Modified a_w	Temperature (°C)
Control	-	0.98	25 20 15
Prochloraz	100 ppb		25
Oil of Thyme	200 ppb	0.96	
Butylated hydroxyanisole (BHA)	50 ppm + 333 ppb of EtOH	0.94	
Control EtOH	333 ppb		

Results

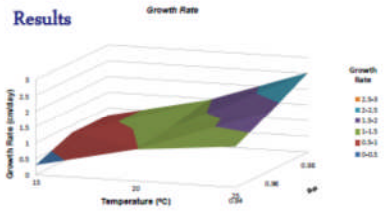


Fig 1. *F. graminearum* growth rate in relation to different a_w x temperature conditions

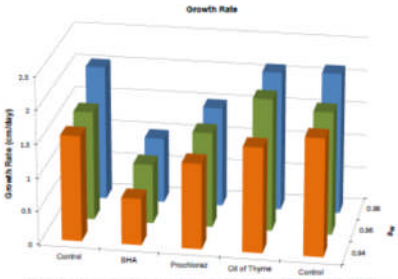


Fig 2. Influence of anti-fungal compounds on growth of *F. graminearum* under different water stress conditions

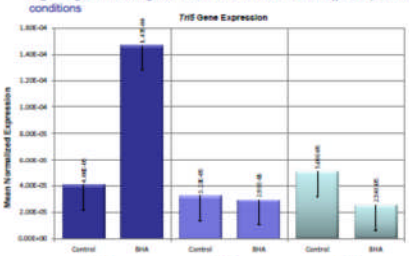


Fig 3. Influence of a_w and BHA (50 ppm) on *Tri5* gene expression at different a_w levels.

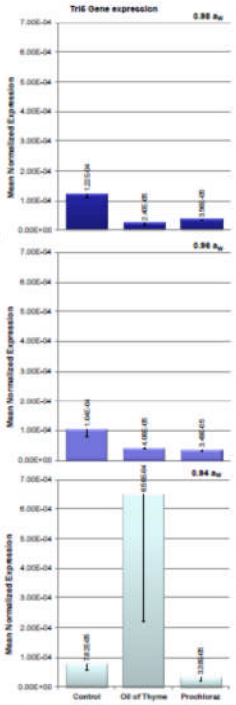


Fig 4. *Tri5* gene expression in relation to antifungal compounds at three different a_w levels.

Conclusions

- Growth rate and *Tri5 gene expression* were affected by both anti-fungal compounds and the environmental factors.
- *Tri5* gene expression was not directly correlated with growth rate.
- Prochloraz was able to reduce both growth rate and *Tri5* gene expression at all water stress conditions.
- *Tri5* gene expression suggests that DON production can be enhanced by the some anti-fungal compounds depending on the water stress imposed.

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Appendix

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Optimization of the total RNA extraction from mycotoxigenic fungi using a bead beating protocol

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Introduction

- Grinding the mycelium with the mortar and pestle using liquid nitrogen is "the" method to extract RNA from filamentous fungi, including mycotoxigenic species as *Aspergillus*, *Penicillium* and *Fusarium*.
- The mortar and the pestle method is time consuming, requires large sample size, a high degree of manual manipulation, and gives overall, a low efficiency of extraction, which also varies with operator expertise.
- Due to the composition of filamentous fungal cell walls, no protocol has been designed to use high speed bead beaters, to the highest possible yields of intact RNA.

✓ The aim of this study was to develop a total RNA extraction method using a bead beating protocol, using a high speed bead beater homogeniser.

Methods/Materials

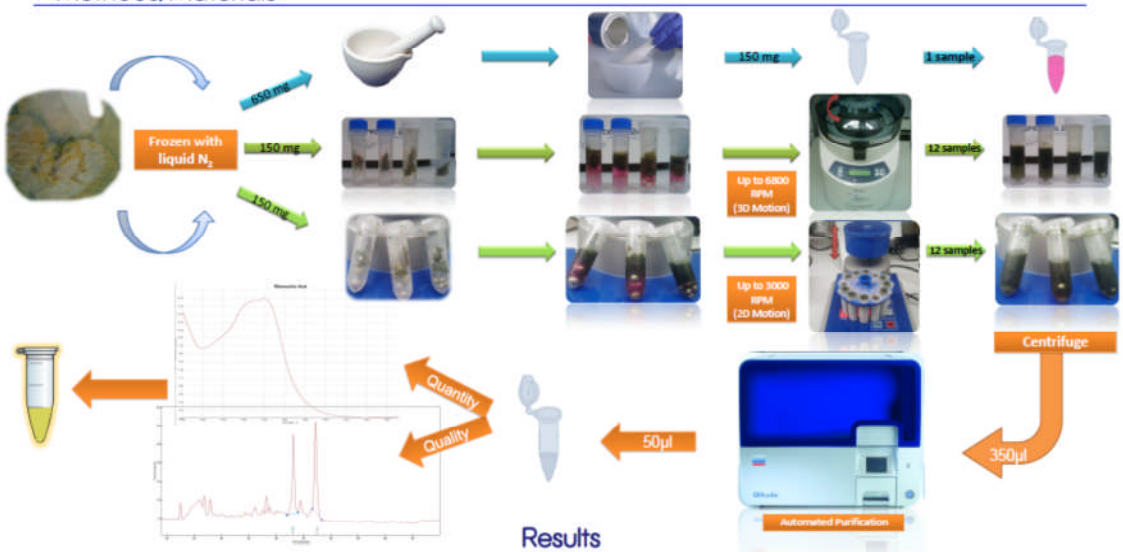

Table 1. Different beads used with the bead homogenizer to extract total RNA from filamentous fungi.

Beads	Material	Size (mm)
VK 01	Glass	0.1
VK 05		0.5
CK 14	Zirconium Oxide (ceramic)	1.4
CK 28		2.8
TC 3	Tungsten Carbide	3.0
SS 5	Stainless Steel	5.0
SS 7		7.0

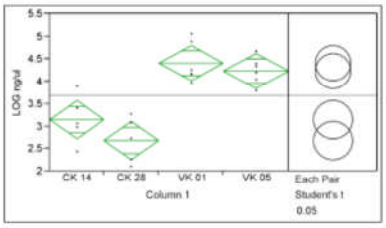

Figure 2. Representation of the ANOVA analysis of bead treatments, including the Student's t comparison of means, of Log ng/µl using different beads by different operators at different times (p-value=00.5). Within a confidence interval of 95% there was no significant different between operators or runs.

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Results

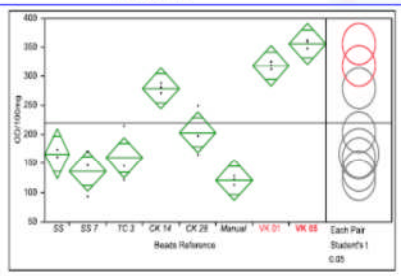

Figure 1. Representation of the ANOVA analysis of bead treatments including "post-hoc" Students's t comparison of means, of OD values/100mg of biomass using the different beads as factors. (p-value=0.05)

Table 2. Homogeneous groups obtained by "post-hoc" Students's t comparison of means. Treatments with the same letters are not significantly different.

Level				Mean OD/100mg
VK 05	A			356.0
VK 01	A	B		317.7
CK 14		B		278.3
CK 28			C	203.4
SS 5			C D	166.8
TC 3			C D	160.1
SS 7			D	136.6
Manual			D	121.0

Conclusions

- The high speed beating associated with glass beads produced the best RNA yields even using reduced sample size.
- The proposed method offers very good inter and intra-day repeatability.
- No significant different between operators or runs were observed, giving good reproducibility.
- This protocol enables 12 samples to be processed at once and reduces the time necessary to extract RNA from filamentous fungi as well as minimizing human error.

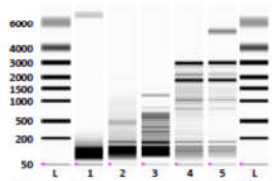

Figure 3. Virtual gel generated by BioRad Experion for samples of different quality (1-5). **Lane 1** appears to possess un-denatured RNA, **Lane 2 and 3** degraded RNA and **Lane 4 and 5** have a RQI above 7.5 (Good Quality). **Lane 5** shows some genomic DNA contamination.

Acknowledgements

A. Medina wants to acknowledge funding from the 7th Framework Program from the European Union, DP3-Support for training and career development of researchers-Marie Curie Actions (Project: PIEF-GA-2009-253014.

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Study of the growth boundaries of 17 spoilage fungi of beverages

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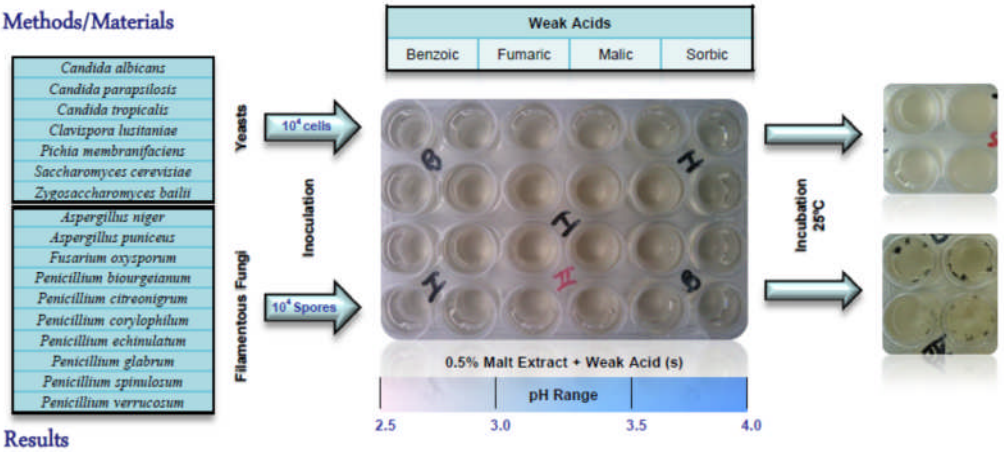
Introduction

- Spoilage of foodstuff is an important economic factor which affects both industry and the consumer.
- The consumer desires more "naturally" preserved products and there is thus pressure to understand the need for preservatives to maintain microbiological shelf life of food.
- There is thus interest in understanding how environmental factors and preservative mixture may affect growth of food spoilage moulds in industry.

The objective of this study was to examine the impact that mixtures of food preservatives have on growth of key food spoilage moulds which have been a problem in the beverage industry under different abiotic factors.

Methods/Materials



Results

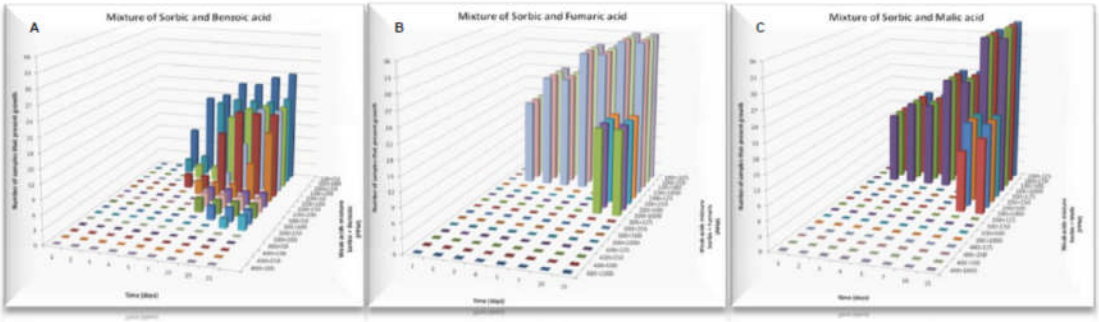


Figure 1. Growth/No Growth boundaries for different fungal strains/species (18; 17 respectively challenged with a mixture of : (A) Sorbic (100, 200, 300 and 400 ppm) and Benzoic acid (50, 100, 150 and 200 ppm); (B) Sorbic (100, 200, 300 and 400 ppm) and Fumaric acid (125, 250, 500 and 1000 ppm); (C) Sorbic (100, 200, 300 and 400 ppm) and Malic acid (125, 250, 500 and 1000 ppm).

	pH	Days							
		1	2	3	4	5	7	10	15
HCl adjusted pH	2.5	74%	85%	91%	94%	94%	94%	94%	96%
	3.0	98%	100%	100%	100%	100%	100%	100%	100%
	3.5	100%	100%	100%	100%	100%	100%	100%	100%
	4.0	100%	100%	100%	100%	100%	100%	100%	100%
KH-Phthalate Buffered pH	pH	Days							
		1	2	3	4	5	7	10	15
	2.5	37%	56%	56%	56%	56%	56%	56%	56%
	3.0	61%	74%	80%	80%	81%	81%	81%	85%
	3.5	94%	100%	100%	100%	100%	100%	100%	100%
	4.0	100%	100%	100%	100%	100%	100%	100%	100%

Table 1. Percentage of all species/strains which grew at different pH treatments.

Conclusions

- All fungi were able to grow at pH 3 when adjusted using HCl.
- The use of single preservatives was not sufficient to prevent growth even at concentrations above the legal limit.
- The use of combined preservatives showed remarkable reduction in the capacity for growth.
- The use of alternative weak acids in conjunction with sorbic acid displayed good inhibition of beverage spoilage fungi, although there appears to be some correlation, especially with concentrations of sorbic acid.

Benzoic (PPM)	Days							
	1	2	3	4	5	7	10	15
0	100%	100%	100%	100%	100%	100%	100%	100%
50	33%	80%	94%	94%	100%	100%	100%	100%
100	33%	74%	89%	94%	100%	100%	100%	100%
150	33%	57%	87%	87%	100%	100%	100%	100%
200	33%	44%	70%	70%	98%	100%	100%	100%
Sorbic (PPM)	Days							
	1	2	3	4	5	7	10	15
0	98%	100%	100%	100%	100%	100%	100%	100%
100	44%	78%	100%	100%	100%	100%	100%	100%
200	33%	61%	89%	94%	100%	100%	100%	100%
300	33%	44%	61%	89%	100%	100%	100%	100%
400	22%	33%	44%	50%	89%	100%	100%	100%

Table 2. Percentage of all species/strains which grew in single preservative treatments at pH 3.

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